

Spectroscopic Studies on Metallothionein Substituted with Cobalt(II) and Iron(II)

Inaugural-Dissertation

zur Erlangung der Philosophischen Doktorwürde
vorgelegt der
Philosophischen Fakultät II
der Universität Zürich

von
MEINRAD ALBERT GOOD
aus Mels SG

Begutachtet von Herrn Prof. Dr. J. H. R. Kägi

Zürich 1987
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Dedicated to
my parents and Bea

Wissenschaftliche Theorien können niemals eine vollständige und definitive Beschreibung der Wirklichkeit liefern. Sie werden stets nur Annäherungen an das wahre Wesen der Dinge sein. Um es ganz grob zu sagen: Wissenschaftler befassen sich nicht mit der Wahrheit, sie befassen sich mit begrenzten und annähernden Beschreibungen der Wirklichkeit.

F. Capra

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S U M M A R Y

Metallothioneins (MT's) are unique low molecular weight (M_r 6000-7000) metal- and cysteine-rich proteins characterized by two tetrahedral tetrathiolate clusters containing three and four metal ions, respectively. Naturally occurring proteins usually contain the diamagnetic metal ions Zn(II) and/or Cd(II). This thesis deals with the substitution of these postransition metal ions by paramagnetic Co(II) and Fe(II) and with the investigation of the spectral properties of these metal derivatives.

Chapter 1 deals with the stepwise incorporation of Co(II) into metal-free MT (apoMT) by monitoring the emergence of electronic absorption, magnetic circular dichroism (MCD) and natural circular dichroism (CD) spectra. In particular the spectral changes attending the transformation from clustered and nonclustered structures when more than 3 to 4 Co(II) equiv are incorporated into apoMT are documented and discussed. The principal finding is that the spectral changes in the d-d region are mainly conditioned by changes in distortion of the Co(II)-sites whereas the spectral properties in the charge-transfer region reflect the interconversion of terminal into bridging thiolate ligands when the clustered structure is formed. The transformation of terminal into bridging ligands has no influence on the ligand field strength and the Racah parameter. However, the bathochromic shift of the nearest charge-transfer bands upon bridge-formation is thought to reflect a reduction in the optical electronegativity of the threefold coordinated thiolate ligand. Characteristic

features in the MCD spectra upon cluster formation are the linear increase of the MCD band at 750 nm and the linear decrease of the MCD band at 680 nm. The most conspicuous features in the CD spectra of the clustered structures are the derivative-shaped ellipticity bands in the charge-transfer and d-d region. Another result of this comparative study is that antiferromagnetic exchange of the vicinal Co(II) in $\text{Co(II)}_7\text{-MT}$, established by electron paramagnetic resonance (EPR) measurements, has no influence on the shape of the electronic absorption, MCD and CD spectra. This is demonstrated by the spectroscopic properties of $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ and $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$. These species containing a single paramagnetic Co(II)-site within a Zn(II)-thiolate or Cd(II)-thiolate cluster matrix exhibit Co(II)-thiolate spectra resembling those of antiferromagnetically coupled $\text{Co(II)}_7\text{-MT}$. The sole differences pertain to the intensity of certain bands in electronic absorption, MCD and CD spectra of $\text{Co(II)}_7\text{-MT}$ when compared to the two hybriide structures. The intensity deficit in the MCD spectrum of $\text{Co(II)}_7\text{-MT}$ was attributed to a partial loss of the C-term due to antiferromagnetic exchange coupling in $\text{Co(II)}_7\text{-MT}$.

The spectroscopic properties of the Co(II)- and Cd(II)-substituted C-terminal segment of rabbit liver MT-1 (α -fragment) is presented in Chapter 2. The α -fragment was obtained by limited proteolysis with subtilisin of partially reconstituted $\text{Cd(II)}_4\text{-MT}$. Electronic absorption, MCD and EPR measurements were employed to monitor the stepwise incorporation of Co(II) into the metal-free fragment (apofragment). Absorption and MCD spectra of the apofragment containing the first 3 Co(II) equiv show the typical features of tetrahedral tetrathiolate Co(II) coordination. However, in

the d-d region only small changes in the visible and no apparent change in the near-infrared region are discernable when the fourth Co(II) equiv is bound. This unusual spectral behaviour was not seen in Co(II)-substitution of native MT and may indicate a difference in cluster geometry. In the charge-transfer region, the binding of all 4 Co(II) equiv is accompanied by characteristic increments of the thiolate S→Co(II) charge-transfer bands. As in the formation of Co(II)₇-MT, the development of the charge-transfer and EPR spectral properties upon binding of the first 2 Co(II) equiv to the apofragment is indicative of noninteracting tetrahedral Co(II) tetrathiolate complexes. The binding of the additional Co(II) ion is accompanied by a red shift in the charge-transfer region and by a loss of paramagnetism reflected by a loss of EPR signals, both diagnostic of the formation of metal thiolate cluster structures. Thus, these data suggest that the four metal cluster in the isolated α -fragment and in Co(II)₇-MT are similar but not identical. Unusual differences between the α -fragment and the complete protein was also noticed in CD titration with Cd(II).

Chapter 3 documents the incorporation of Fe(II) into apoMT. The electronic absorption spectrum of the fully metal occupied Fe(II)₇-MT is typical of tetrahedral tetrathiolate Fe(II) coordination. This is documented by the presence of a ligand field $^5E \rightarrow ^5T_2$ transition in the near-infrared region centered at about 1850 nm ($\epsilon_{Fe} \approx 100 \text{ M}^{-1} \text{ cm}^{-1}$) and a broad charge-transfer absorption in the UV-region with a shoulder at 314 nm. A metal-thiolate cluster structure is inferred from the 7-to-20 ratio of metal ions to cysteine residues and from spectral studies in which successive increments of Fe(II) were incorporated into the metal-free protein. Thus, up to about 4 equiv

the charge-transfer absorption, MCD and CD features of the complexes formed resemble closely those of reduced rubredoxin from *Desulfovibrio gigas* for which tetrahedral tetrathiolate Fe(II) coordination is reported. However, upon further addition of Fe(II), the charge-transfer absorption bands undergo a progressive red shift until the full metal occupancy of 7 Fe(II) per molecule is reached. The bathochromic shift which is also manifested in the MCD spectra can be ascribed to the transformation of some of the terminal thiolate ligands to bridging when the full complement of Fe(II) is bound. The concomitant loss in amplitude of the MCD bands above 4 equiv is thought to arise from exchange coupling of vicinal Fe(II) via thiolate bridges. This interpretation is supported by the observation that the intensities of the MCD bands, when referred to Fe(II) content, are larger in $\text{Fe(II)}_1\text{Zn(II)}_6\text{-MT}$ than in $\text{Fe(II)}_7\text{-MT}$. The inversion of the CD signals in $\text{Fe(II)}_7\text{-MT}$ and $\text{Fe(II)}_1\text{Zn(II)}_6\text{-MT}$ compared to isolated FeS_4^{2-} -sites in Fe(II)-MT is thought to be characteristic for the clustered structure in this Fe(II)-substituted protein. The spectroscopic data suggest that the metal ions in $\text{Fe(II)}_7\text{-MT}$ are organized similarly to those found in native MT. Moreover, $\text{Fe(II)}_7\text{-MT}$ is the first Fe(II)-thiolate cluster structure in a biological molecule thus far reported.

Z U S A M M E N F A S S U N G

Metallothioneine (MT) sind niedermolekulare Proteine (M_r 6000-7000), welche einen hohen Metallgehalt und einen hohen Anteil an Cystein aufweisen. Die aus Säugergeweben isolierten Proteine bestehen aus zwei Domänen, die von zwei sogenannten Metallthiolatclustern gebildet werden. Die Metallthiolatcluster sind aus 3 bzw. 4 bivalenten Schwermetallionen und 9 bzw 11 Thiolatliganden von Cysteinseitenketten aufgebaut. In den natürlich vorkommenden Metallothioneinen werden hauptsächlich Zn(II) und Cd(II) gebunden, welche ausschliesslich tetraedrisch via Cysteinschwefel koordiniert sind. Die vorliegende Arbeit beschäftigt sich mit der Substitution von diesen diamagnetischen Metallionen durch paramagnetische Co(II) und Fe(II) Ionen. Die Metallderivate wurden mit verschiedenen spektroskopischen Techniken untersucht.

In Kapitel 1 wird der schrittweise Einbau von Co(II) in das metallfreie Metallothionein (ApoMT) spektroskopisch durch Messung der elektronischen Absorption, des natürlichen Circular dichroismus (CD) und des magnetischen Circular dichroismus (MCD) untersucht. Das Hauptergebnis ist, dass die spektralen Eigenschaften dieser Co(II)-substituierten Derivate in der d-d Region vorwiegend auf Änderungen der Verzerrung der Co(II)-Bindungsstellen, und in der Charge-Transfer Region auf die Umwandlung von terminalen Thiolatliganden in Brückenthioatliganden zurückgeführt werden können, wenn die Clusterstruktur gebildet wird. Es erwies sich dabei, dass die chemischen bzw. physikalischen Änderungen in

der Molekel keinen signifikanten Einfluss auf die Ligandenfeldaufspaltung und den Racah Parameter ausüben. Im Gegensatz dazu lässt die Rotverschiebung der Charge-Transfer Banden beim Uebergang von terminalem Schwefel zu Brückenschwefel auf eine Änderung der optischen Elektronegativität der Liganden schliessen. Die MCD Spektren zeigen beim Aufbau der Clusterstruktur eine charakteristische Veränderung in den 750 und 650 nm Banden. Bei der Co(II)-Titration nimmt oberhalb von Co(II)₄-MT die MCD Bande bei 750 nm linear zu, während die MCD Bande bei 680 nm linear abnimmt. In den CD-Spektren ist das Hauptmerkmal der verbrückten Komplexe die Aufspaltung der einfachen CD Banden im d-d Bereich in Doppelbanden mit entgegengesetzter Polarisierung. Es konnte weiter gezeigt werden, dass der in Co(II)₇-MT vorhandene antiferromagnetische Austausch zwischen den benachbarten Co(II) Ionen keinen Einfluss auf die Form und die Bandenlage der entsprechenden Absorptions-, MCD- und CD-Banden ausübt. Dies konnte aus den spektroskopischen Eigenschaften von Co(II)₁Zn(II)₆-MT und Co(II)₁Cd(II)₆-MT geschlossen werden, wo Co(II) in einer spektroskopischen Matrix von Zn(II)₆-MT bzw. Cd(II)₆-MT eingebaut ist. Werden diese Hybride und Co(II)₇-MT miteinander verglichen, so findet man grosse Unterschiede in der Intensität der entsprechenden Absorptions-, MCD- und CD-Banden. Die Intensitätsunterschiede in den MCD Spektren können möglicherweise dadurch erklärt werden, dass durch die antiferromagnetische Koppelung der Co(II) Ionen in Co(II)₇-MT der Anteil des C-Terms im MCD Spektrum erniedrigt wird.

Um weitere spektroskopische Informationen zu MT zu erhalten, wurde die C-terminale Domäne (α -Fragment) von MT, welche vier Metalle bindet, isoliert. Dieses MT-Derivat wurde durch enzymatische Abspaltung (Subtilisin) von partiell rekonstituiertem Cd(II)_4 -MT in der Form des Cd(II)_4 -Fragments rein erhalten. Die spektroskopischen Eigenschaften des Co(II) -substituierten Fragments werden in Kapitel 2 diskutiert und mit Co(II)_7 -MT verglichen. Absorptions und MCD Spektren von Apofragment, welches 3 Co(II) Äquivalente enthält, zeigen, dass diese Ionen tetraedrisch von vier Schwefelliganden der Cysteine koordiniert werden. Bei der Bindung des vierten Co(II) zeigt sich das charakteristische Merkmal einer Intensitätszunahme und einer Rotverschiebung in der Charge-Transfer Region. Keine nennenswerte spektralen Änderungen werden dagegen in der d-d Region beobachtet. Dieses ungleiche spektrale Verhalten wird bei Co(II)_7 -MT nicht beobachtet. Die spektralen Daten weisen darauf hin, dass das vierte gebundene Co(II) eine pseudooktaedrische Koordination hat und somit das Co(II)_4 -Fragment eine andere Clustergeometrie aufweist. Ungewöhnliche spektrale Unterschiede zwischen dem α -Fragment und ApoMT konnten auch bei Titration mit Cd(II) in den resultierenden CD Spektren beobachtet werden. Diese Daten demonstrieren, dass der 4-er Cluster im isolierten α -Fragment und im Holoprotein ähnlich aber nicht gleich ist.

Um festzustellen, ob der charakteristische Einbau von Co(II) in ApoMT auf dieses Metallion beschränkt ist, wurde ein weiteres paramagnetisches Metallion verwendet. Hierzu bot sich Fe(II) sowohl aus spektroskopischen als auch aus biologischen Gründen an. Obwohl Eisen-Schwefel Verbände in biologischen Systemen weit verbreitet sind, konnten bis jetzt nur geringe

Mengen von diesem Metallion in nativem MT nachgewiesen werden. Kapitel 3 dokumentiert die spektroskopischen Eigenschaften des Fe(II)-substituierten Metallothioneins. Das Absorptionsspektrum des voll besetzten Fe(II)₇-MT zeigt die typischen Merkmale von tetraedrisch koordiniertem Fe(II) mit Schwefelliganden. Dies wird durch die Anwesenheit einer Ligandenfeld-Bande (d-d Uebergang) im nahen Infrarot bei ungefähr 1850 nm mit einem molaren Extinktionskoeffizienten von $100 \text{ M}^{-1} \text{ cm}^{-1}$ pro Fe(II) bewiesen. Das Absorptionsspektrum in der UV-Region weist die charakteristischen Eigenschaften von Fe(II)→Schwefel Charge-Transfer Uebergängen auf. Aufgrund der Stoichiometrie von 7 Metallen pro 20 Cysteine und des schrittweisen Einbaus von Fe(II) in ApoMT wird eine Clusterstruktur vorgeschlagen. Die ersten vier gebundenen Fe(II)-Äquivalente in Fe(II)-MT zeigen die typischen spektralen Eigenschaften in den Absorptions-, MCD- und CD- Spektren von tetraedrisch koordiniertem Fe(II) mit terminalen Schwefelliganden. Diese Spektren haben eine grosse Ähnlichkeit mit denjenigen von reduziertem Rubredoxin aus *Desulfovibrio gigas*, wo Fe(II) tetraedrisch an vier Cysteine koordiniert ist. Weitere Zugabe von Fe(II) bis zum voll besetzten Fe(II)₇-MT manifestiert sich in einer Rotverschiebung der Charge-Transfer Banden. Diese bathochrome Verschiebung, welche ebenfalls in den MCD Spektren ersichtlich ist, wird auf das Auftreten von Brückenschwefelliganden zurückgeführt. Der gleichzeitige Verlust der Intensität der MCD Amplituden oberhalb Fe(II)₄-MT kann möglicherweise durch die antiferromagnetische Koppelung von benachbarten Fe(II) Ionen bewirkt sein. Diese Erklärung wird durch die Tatsache unterstützt, dass die MCD Amplituden von Fe(II)₁Zn(II)₆-MT wesentlich grösser sind als

diejenigen von $\text{Fe(II)}_7\text{-MT}$. Die Umkehrung der CD-Signale in $\text{Fe(II)}_7\text{-MT}$ und $\text{Fe(II)}_1\text{Zn(II)}_6\text{-MT}$ im Gegensatz zu isolierten FeS_4^{2-} -Bindungsstellen in Fe(II)-MT scheint ebenfalls ein Charakteristikum der Clusterstruktur von $\text{Fe(II)}_7\text{-MT}$ zu sein. Diese spektroskopischen Daten zeigen, dass Fe(II) in $\text{Fe(II)}_7\text{-MT}$ ähnlich gebunden ist wie die Metallionen der Gruppe 2 B. Es ist erwähnenswert, dass $\text{Fe(II)}_7\text{-MT}$ die erste Eisen-Clusterverbindung eines biologischen Moleküls ist, welches als Liganden ausschliesslich Thiolatschwefel enthält.

GENERAL INTRODUCTION

Metallothioneins (MT's) represent a class of low molecular weight (M_r 6000-7000) metal and sulfur rich proteins, which are widely distributed in nature (Kägi et al., 1984). These ubiquitous proteins occur in many mammalian species as well as in eukaryotic microorganisms (Prinz & Weser, 1975; Lerch, 1980), prokaryotes (Higham et al., 1984) and in some plants (Rausser & Curvetto, 1980; Grill et al., 1985). All mammalian forms examined to date consist of a single polypeptide chain with 61 or 62 amino acids of which 20 residues are cysteine and contain seven diamagnetic metal ions such as Zn(II) and/or Cd(II). Another characteristic feature is the absence of aromatic amino acids. Typical of all MT's is the occurrence of unique Cys-X-Cys sequences in their primary structures, where X corresponds to an amino acid residue other than Cys. There are two major electrophoretically separable species present in most mammals, MT-1 and MT-2, which have closely related but distinct chemical structures (Kägi et al., 1984). The two differently charged forms can be resolved into further distinct molecular subforms by using high performance liquid chromatography (Kägi et al., 1984; Hunziker, 1986).

Spectroscopic studies have established that the seven metal ions are coordinated by 20 cysteine-sulfur ligands with overall T_d -type symmetry (Vasak, 1980; Vasak & Kägi, 1981). ^{113}Cd -NMR studies have shown that the metal ions in the polypeptide chain are arranged in two separate metal clusters of three and four metals, respectively (Otvos & Armitage, 1980). This cluster architecture is supported by the demonstration that the

four-metal domain can be isolated after proteolytic digestion with subtilisin (Winge & Miklossy, 1982). The latter studies also indicated that the four-metal cluster (α -fragment) is formed by the C-terminal part whereas the three metal cluster (β -fragment) is made up of the N-terminal part of the polypeptide chain. Recently, models for the 3D solution structure of rabbit liver $\text{Cd(II)}_7\text{-MT-2}$ as determined by 2D NMR (Frey et al., 1985, Braun et al., 1986) and for the crystal structure of rat liver $\text{Zn(II)}_2\text{Cd(II)}_5\text{-MT-2}$, as determined by X-ray diffraction (Furey et al., 1986), have been put forward. Although the X-ray structure and the 2D NMR solution structure remain at variance in certain details of the peptide folding and metal binding their overall organization is identical.

An interesting feature of MT is that their biosynthesis is induced by the administration of a variety of agents among them by heavy metal ions. It is believed, therefore, that MT's are involved in trace metal metabolism and storage and detoxification of essential and nonessential d^{10} metal ions (Nordberg & Kojima, 1979). The abundance of Zn(II) in the protein makes it likely that the protein exerts its primary role in relation to this important trace element. However, its high affinity for Cd(II) and Hg(II) predisposes it also to a protective function in poisoning by these metals. Moreover, the well documented induction of MT by glucocorticoid hormones (Failla & Cousins, 1978; Karin & Herschman, 1979) and interferon (Friedman & Stark, 1985) and interleukin 1 (Karin et al, 1985) implies that this protein is involved also in other as yet unknown physiological functions. Bakka & Webb (1981) demonstrated that mammalian cells with a high level of MT's display increased resistance to the damaging effects of X-rays. This finding

is in agreement with the recent observation that MT's can act as efficient scavengers of free hydroxyl radicals in vitro (Thornally & Vasak, 1985). In view of these many possible functions it is noteworthy that in most vertebrate species examined to date MT's are encoded by a multigene family and that individual members of the MT gene family can display different responses to inducers because they are regulated by distinct promoter elements (for a review see Hamer, 1986).

In recent years MT has attracted also the attention of spectroscopists. MT can be viewed as a unique spectroscopic model for metal-thiolate clusters. An interesting feature of these proteins is the capacity that the seven bivalent metal ions of the native forms can be substituted stoichiometrically by a variety of other metal ions (Kägi et al., 1984). Co(II) is a transition metal ion which has been widely used as a spectroscopic probe for Zn(II)-metalloproteins (Vallee & Holmquist, 1980; Bertini & Luchinat, 1983; Bertini et al., 1986). Because of the similarity of Co(II) and Zn(II) in many chemical properties Co(II) can replace Zn(II) in Zn(II)-metalloproteins while retaining the same coordination geometry. This has been demonstrated by the preservation of the enzymatic activity of Co(II)-substituted Zn(II)-enzymes. In some cases the activity is even higher than in the corresponding native Zn(II)-enzymes (Vallee & Holmquist, 1980). Owing to its completely filled d-shell (d^{10} configuration) the optical features of Zn(II) are limited to the high energy region. In contrast, Co(II) has a partially filled d shell (d^7 configuration) which is responsible for many electronic properties of this paramagnetic metal ion. The so-called d-d transitions, observed in the visible and near-infrared region, are very

sensitive to coordination geometry and to the type of ligands. Additional information of such Co(II)-substituted metalloproteins can be obtained from electron paramagnetic resonance (EPR) measurements. It has been shown that such studies are useful in distinguishing magnetically coupled Co(II) ions in the clustered structure of Co(II)-MT from the isolated Co(II)-sites in nonclustered complexes (Vasak & Kägi, 1981).

The major goal of my thesis was to gain further insight into the cluster formation of Co(II)-substituted MT using electronic absorption, magnetic circular dichroism (MCD), circular dichroism (CD) and EPR techniques. Similar studies were also conducted with MT into which paramagnetic Fe(II) (d^6 configuration) was substituted as a spectroscopic probe. This successful incorporation of Fe(II) into MT opened interesting new perspectives not only on the structural and spectroscopic features of this complex but may also have bearing on a possible role for MT in iron metabolism.

The work is divided into three chapters. Chapter 1 deals with the cluster formation studies of Co(II)-substituted MT, chapter 2 focuses primarily on the spectroscopic properties of the Co(II)-substituted α -fragment of MT and chapter 3 concerns the spectroscopic properties of the Fe(II)-thiolate clusters in Fe(II)₇-MT.

C H A P T E R 1

SPECTROSCOPIC PROPERTIES OF CLUSTER FORMATION IN COBALT(II)-SUBSTITUTED METALLOTHIONEIN

1-1 Introduction

Earlier studies of Co(II)-substituted MT have revealed the same 7:1 metal-to-protein ratio as found in native Zn(II)- and/or Cd(II)-MT (Vasak, 1980; Vasak & Kägi, 1981). Using electronic absorption and EPR techniques, these authors found that in Co(II)-MT the metal ions are tetrahedrally coordinated to four cysteine thiolate ligands. Moreover, EPR and magnetic susceptibility measurements revealed antiferromagnetic coupling between vicinal Co(II) proving the existence of clustered structures. By EPR titration studies it was shown also that such oligonuclear complexes are formed only when at least 3 to 4 Co(II) equiv are bound to the protein. Therefore, it was concluded that under the conditions employed the first 3 to 4 Co(II) equiv bound in Co(II)-MT occupy non-interacting separate metal binding sites, each made up of four terminal thiolate ligands.

The aim of the work reported and discussed in this chapter was to gain a better understanding about the physical basis leading to the spectral changes in electronic absorption, MCD and CD spectra on passing from isolated tetrathiolate Co(II)-complexes of incompletely saturated Co(II)-MT to the clustered structure in metal saturated Co(II)₇-MT. MCD spectroscopy

is an important tool in elucidating the coordination geometry in Co(II)-substituted proteins (Vallee & Holmquist, 1980). MCD and CD are quite unrelated phenomena although both are monitored by the same experimental means. Whereas MCD is an intrinsic property of the electronic configuration of a chromophore CD depends on molecular chirality. CD features are, therefore, very sensitive to the generally dissymmetric protein environment whereas MCD features are not. Because of their complex origin CD spectra are less amenable to theoretical analysis.

In this chapter changes in electronic absorption, MCD and CD features attending the transition of Co(II)-MT from the nonclustered to the clustered forms are documented and discussed. In addition, data are reported on the spectroscopic properties of $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ and $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$, where Co(II) is incorporated in a spectroscopically silent matrix of $\text{Zn(II)}_6\text{-MT}$ and $\text{Cd(II)}_6\text{-MT}$, respectively. Both, theoretical and empirical arguments are utilized to interpret the wealth of spectral data now available on the title complex.

1-2 Results and Discussion

The changes attending the stepwise incorporation of Co(II) into apoMT were monitored by electronic absorption, MCD and CD spectroscopy. The data obtained are divided into three parts. The first part deals with the d-d region, the second with the charge-transfer region of spectra of solely Co(II) containing forms of MT whereas in the third part spectroscopic properties of Co(II) in a matrix of $\text{Zn(II)}_6\text{-MT}$ and $\text{Cd(II)}_6\text{-MT}$ are presented.

LOW ENERGY (d-d) REGION

In a tetrahedral ligand field Co(II) exhibits three spin-allowed d-d transitions designated as ν_1 , ν_2 and ν_3 , respectively (Figure 1-1). The sevenfold degenerate ^4F ground term of the d^7 Co(II) ion is split into the three levels $^4\text{T}_1$, $^4\text{T}_2$ and $^4\text{A}_2$ of which the first two levels are threefold degenerate and the last level is nondegenerate. The $\nu_1[{}^4\text{A}_2 \rightarrow {}^4\text{T}_2(\text{F})]$ and $\nu_2[{}^4\text{A}_2 \rightarrow {}^4\text{T}_1(\text{F})]$ transitions are observed in the near-infrared whereas the $\nu_3[{}^4\text{A}_2 \rightarrow {}^4\text{T}_1(\text{P})]$ transition is located in the visible region. Due to spin orbit coupling the ν_3 -transition usually exhibits a fine structure. In a spectroscopic study of Co(II) in the crystal lattice of ZnS or CdS the four most intense peaks of the ν_3 -transition were shown to originate from splitting of the $^4\text{T}_1(\text{P})$ excited state by spin orbit coupling into four sublevels E' , $3/2 \text{ U}'$, E'' and $5/2 \text{ U}'$ (Weakliem, 1962). The ν_1 -transition of Co(II)-MT, expected by ligand field calculations (see below) to occur below

2000 nm, cannot be observed because it is obscured by the vibrational transitions of water. Hence, in this first part data and discussions will be restricted to manifestations of the various mono- and oligonuclear complexes in Co(II)-MT in electronic absorption in the ν_2 and ν_3 region and the alterations of MCD and CD signals associated with the ν_3 -band.

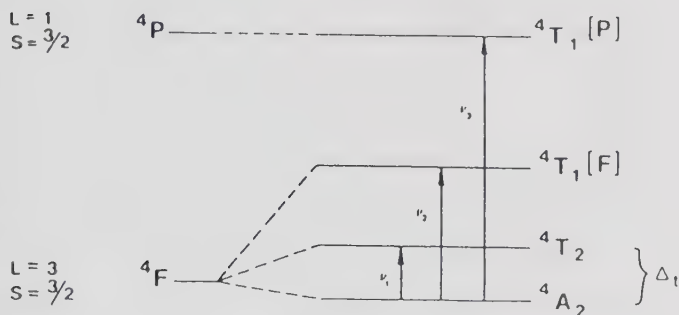


Figure 1-1: Simplified energy level diagram for Co(II) in a tetrahedral ligand field ignoring spin orbit coupling. The three transitions are indicated by arrows.

(A) **Electronic Absorption Properties.** Figure 1-2 shows electronic absorption spectra of Co(II)-MT of increasing intensity formed at various Co(II)-to-protein ratios. The position and shape of the ν_2 -band in the near-infrared region (Figure 1-2, C) remain unchanged throughout titration whereas the bands of the ν_3 -transition (Figure 1-2, B) preserved their shape only up to 4 Co(II) equiv. This disparity is illustrated also in Figure 1-5 (top) where the absorbancy at 1275 nm and 680 nm are plotted as a

function of Co(II)-to-apoMT ratio, respectively. In contrast to the linear increase of the ν_2 -absorption there is a reduction in the absorption increment beyond 3 to 4 Co(II) equiv when measured at the 680 nm band. Judged from the broadness of its profile the ν_2 -transition is also split by spin orbit coupling. This was observed in tetrahedrally coordinated halide Co(II)-complexes which exhibit a wealth of fine structure in this transition (Cotton et al., 1961). A ν_2 -band with fine structure was also reported for the tetrahedral tetrathiolate Co(II)-complex $[\text{Co}(\text{S}_2\text{-o-xy})]^{2-}$ (Lane et al., 1977). The relatively sharp absorption peak at 1400 nm arises most likely from vibrational overtone transitions either from the protein or from residual HOD. In the spectra of Co(II)₁-MT to Co(II)₄-MT the band maxima of the ν_3 -transition at 730 (sh), 680 and 620 (sh) nm are typical of terminal tetrahedral tetrathiolate Co(II)-complexes (Holah & Coucouvanis, 1975; Lane et al., 1977; Swenson et al., 1978; Dance, 1979; Vasak & Kägi, 1981). Since for most inorganic tetrahedral Co(II)-complexes the spin orbit coupling constant is of the order of 150 cm^{-1} the splitting of the ν_3 -transition of the absorption spectra into three components with an energy separation of about 1000 cm^{-1} and 1420 cm^{-1} cannot be due to spin orbit coupling alone (Cotton et al., 1961). Most likely, the larger separation of the ν_3 -transition into three well resolved bands arises from distortions from regular T_d -symmetry of the Co(II)-sites (Bertini & Luchinat, 1983). In the spectra going from Co(II)₄-MT to the fully metal occupied Co(II)₇-MT the position of the ν_3 -bands is shifted to 748, 690 and 600 (sh) nm. The increased energy separation of 1120 cm^{-1} and 2175 cm^{-1} associated with these shifts probably reflects additional distortion of the Co(II)-sites imposed upon the transition of the isolated metal binding sites to the clustered aggregate.

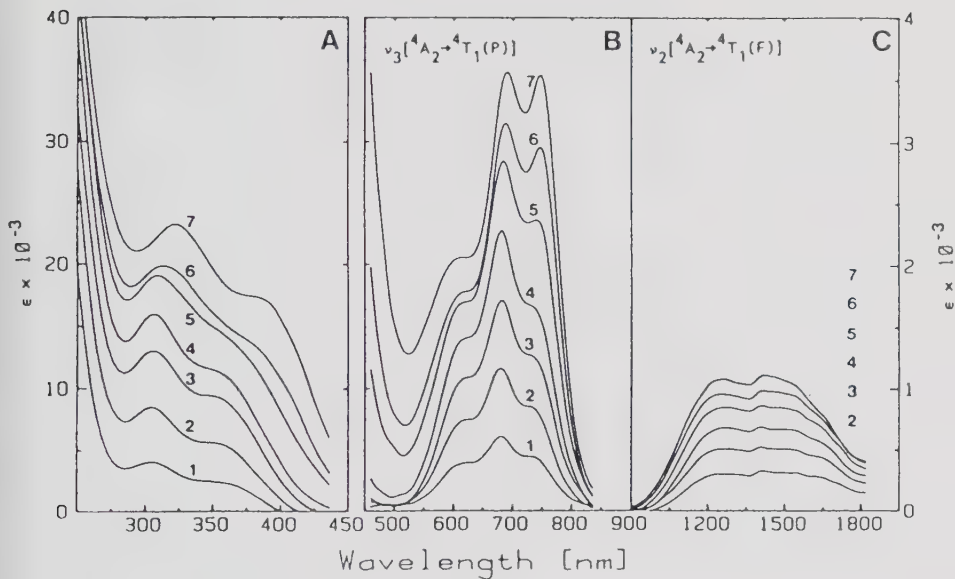


Figure 1-2: Electronic absorption spectra of Co(II)-MT in the charge-transfer (A), visible (B) and near-infrared (C) region as a function of Co(II)-to-protein ratio. The numbers indicate the moles of Co(II) per mole of apoMT. ϵ is based on protein concentration. Conditions: 0.17 mM Co(II)-MT in 160 mM Tris-HCl, pH 8.3, at room temperature. Spectra in the near-infrared region were obtained in D_2O .

The quality of the data obtained permits calculation of the ligand field strength Δ_t and the Racah parameter B' for isolated and for the clustered Co(II)-complexes in Co(II)-MT. Neglecting spin orbit coupling the spectral parameter B' , reflecting electron-electron repulsion of the d electrons in the complex, and Δ_t can be calculated from the frequencies ν_2 and ν_3

$$\nu_2 = 1.5\Delta_t + 7.5 B' - Q \quad (1)$$

$$\nu_3 = 1.5\Delta_t + 7.5 B' + Q \quad (2)$$

where Q is

$$Q = 1/2[(0.6\Delta_t - 15B')^2 + 0.64\Delta_t^2]^{1/2}$$

derived by standard procedures of ligand field theory (Carlin, 1965). Evaluation of the above equations yields a convenient expression for Δ_t and B'

$$\Delta_t^2 - 0.529(\nu_2 + \nu_3)\Delta_t + 0.294(\nu_2 \nu_3) = 0 \quad (3)$$

$$B' = (\nu_2 + \nu_3 - 3\Delta_t)/15 \quad (4)$$

The calculated parameters are summarized in Table I. Note that accuracy of the data for Δ_t and B' is limited since the location of the maximum in the broad ν_2 -band is somewhat arbitrary. Furthermore, a straight forward analysis of the ν_3 -transition was possible only in the case of mononuclear complexes where the Co(II) ions are coordinated solely by terminal sulfur ligands (i.e. in Co(II)₁-MT to Co(II)₄-MT). In the clustered structure (i.e. Co(II)₇-MT) where both terminal and bridging thiolate sulfur ligands are present an overlap of the charge-transfer envelope (see below) with the

absorption profile of the d-d region renders the determination of the weighted maximum of the ν_3 -transition somewhat uncertain. To overcome this problem the three bands of the spin orbit coupling components in the visible and the 390 nm band in the charge-transfer region of $\text{Co(II)}_7\text{-MT}$ were fitted by gaussian analysis. The spectral parameters Δ_t and B' for $\text{Co(II)}_7\text{-MT}$ calculated from these gaussian bands are comparable to those obtained for $\text{Co(II)}_1\text{-MT}$ (Table I). Thus, the ligand field strength and the electron repulsion parameter for terminal and bridging thiolate sulfur ligands are comparable. The Δ_t - values reported for tetrahedral tetrathiolate Co(II) model complexes (Anglin & Davison, 1975; Holah & Coucouvanis, 1975; Lane et al., 1977) are in the range of 3800 cm^{-1} to 4980 cm^{-1} and agree well with those measured in Co(II)-MT . Comparison of this Δ_t - value with those in the common spectrochemical series (Cotton et al., 1961) reveals a close correspondence to that found for NCS complexes.



The nephelauxetic (cloud expanding) parameter, β , which is the ratio of B' to B (the free ion value) provides a measure of the covalency of the Co(II)-thiolate complexes. The low β -value of 0.6 compared to that of the other members of the nephelauxetic series (see below) confirms that sulfur is the most covalently bound ligand.



Table I Experimental Data and Ligand Field Spectral Parameters for Co(II) Complexes with Cysteine Sulfur Ligands

Complex	ν_2 (cm ⁻¹)	ν_3 (cm ⁻¹)	Δ_t (cm ⁻¹)	B' (cm ⁻¹)	β^c
Co ₁ -MT	7900 ^b	15'000 ^a	4697	587	0.605
Co ₇ -MT	7900 ^b	14'800 ^a	4710	571	0.588

a) Data calculated using intensity weighted ν_3 maximum

b) Data calculated using central ν_2 band

c) $\beta = B'/B$, where B = 971 cm⁻¹ (Lever, 1984)

(B) MCD Properties. Characteristic changes with incremental addition of Co(II) are also seen in the MCD spectra of Co(II)-MT (Figure 1-3, bottom). The first 4 Co(II) equiv elicit two large negative MCD bands with comparable magnetic ellipticities at (-) 730 and (-) 680 nm and two low intensity bands with extrema at (+) 616 and (-) 573 nm, respectively. Except for the band at 573 nm, which has no correspondence in the electronic absorption spectrum, these MCD extrema coincide in position with those observed in electronic absorption. In the fully metal occupied Co(II)₇-MT the first two low energy MCD bands are shifted to 750 and 688 nm paralleling the displacement of the corresponding absorption bands (see above). The other two MCD bands are unaffected by the increased metal occupation. Marked alterations are noticeable also in the amplitude of the MCD signal of the 680 and 730 nm bands when the cluster structure is

formed. Thus, the size of the MCD band at 730 nm increases nearly linearly up to full metal occupation in $\text{Co(II)}_7\text{-MT}$ whereas the MCD band at 680 nm increases linearly up to approximately 4 Co(II) equiv but drops again upon further addition of Co(II) . An illustration of this effect is given in Figure 1-5 (bottom) where $-\left[\Theta\right]_{\text{M}}$ at 680 nm is plotted as a function of the Co(II) -to-apoMT ratio. The change from an ascending to a descending slope at approximately 4 Co(II) equiv indicates that cluster formation sets in beyond this Co(II) -to-protein ratio. The origine of these conspicuous spectral changes is not clear. Since the ligand field parameters Δ_{t} and B' characterizing the Co(II) -thiolate complexes do not change significantly upon going from mononuclear to oligonuclear complexes in Co(II) -MT it follows that transformation of terminal into bridging thiolate ligands has no direct spectrochemical effect on the Co(II) d-d transitions. It appears, instead, that the manifestation of cluster formation in electronic absorption and MCD are conditioned more indirectly by changes in spin orbit coupling of the excited state resulting from structural distortion of the tetrahedral Co(II) -thiolate complexes constrained in the clusters. That some changes in the MCD spectrum arise from antiferromagnetic exchange between vicinal Co(II) remains also a possibility.

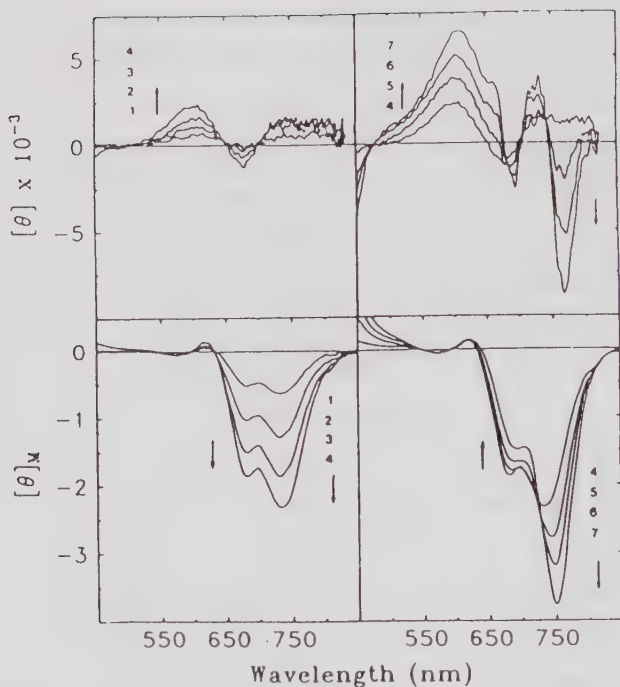


Figure 1-3: CD (top) and MCD (bottom) spectra of Co(II)-MT in the d-d region as a function of Co(II)-to-protein ratio. The numbers indicate the moles of Co(II) per mole of apoMT and the arrows symbolize the development of the bands with increasing amount of Co(II). $[\theta]$ and $[\theta]_M$ are based on the protein concentration. Conditions are as in Figure 1-1.

(C) CD Properties. The effect of varying metal-to-protein ratio on the CD spectra is shown in Figure 1-3 (top). Again, there are marked changes upon going from the nonclustered to the clustered structure. Up to 4 Co(II) equiv two distinct CD bands can be noted at (+) 606 and (-) 680 nm whereas a broad positive band is centered about 770 nm characterizing the isolated tetrahedral tetrathiolate Co(II)-complexes formed in the protein under these conditions. Note, that the spin orbit coupling components of the electronic absorption spectra are located at similar positions (see above). Further addition of Co(II) up to the fully occupied Co(II)₇-MT shows an increase in ellipticity of the CD band at 600 nm. This is illustrated by the plot of the 600 nm CD band as a function of Co(II)-to-protein ratio (Figure 1-5, middle). There is a marked change of the slope between 3 and 4 Co(II) equiv. Moreover, a shoulder appears at 662 nm in the fully metal occupied Co(II)₇-MT. Interestingly the broad CD 770 nm band is split into a derivative-shaped band centered at (+) 732 and (-) 768 nm, respectively (Table II). Due to this band splitting, an isodichroic point emerges at 748 nm, which coincides with the lowest energy electronic absorption maximum of the spin orbit coupling component of Co(II)₇-MT. Such CD behaviour is reminiscent of excitonic coupling of directionally oriented electric dipole moments in vicinal chromophores. However, considering the rather low transition probability of the ν_3 -transition in the electronic absorption spectrum of Co(II)₇-MT ($\epsilon_{748}=510 \text{ M}^{-1} \text{ cm}^{-1}$) such an origine seems very unlikely. Interestingly, similar CD features are observed in the corresponding matrix spectra (see below).

HIGH ENERGY (CHARGE-TRANSFER) REGION

Since apoMT does not absorb appreciably above 250 nm (Vasak & Kägi, 1983) all spectral features of Co(II)-substituted MT arise from charge-transfer transitions or ligand internal excitations affected by the interaction with Co(II) (Mastropolo et al., 1977).

(A) **Electronic Absorption Properties.** Up to 4 Co(II) equiv an absorption profile with a maximum at 305 and a shoulder at 350 nm develops which upon further addition of Co(II) are shifted to 322 and 390 nm, respectively (Figure 1-2, A). These alterations of the positions of the bands are due to the appearance of bridging thiolate ligands when the clustered structure is formed (Vasak & Kägi, 1981). The red shift is reflected also by the increase in incremental absorbancy at 390 nm above 3 to 4 Co(II) equiv (Figure 1-5, top). No significant change in absorbancy occurs above 7 equiv of Co(II) indicating saturation of all metal binding sites in Co(II)₇-MT. Similar bathochromic shifts in electronic absorption have been noticed in the titration of apoMT with diamagnetic Cd(II) and Hg(II) (Gilg, 1985; Willner, 1987; Bernhard, 1986) and with paramagnetic Fe(II) (see Chapter 3). By Jorgensen's semiempirical optical electronegativity theory (Jorgensen, 1970), these shifts can be interpreted as reflecting differences in optical electronegativity between terminal and bridging thiolate ligands. The basic assumption of this theory is that the first charge-transfer transition is related to the electronegativity difference between the donor and acceptor ions

$$\nu_{\text{corr}} = 30'000 \text{ cm}^{-1} (X_{\text{L}} - X_{\text{M}}) \quad (5)$$

where ν_{corr} represents the energy of the lowest charge-transfer transition band corrected for the ligand field strength and the spin-pairing energy parameter D. X_L and X_M are the optical electronegativities of ligand and metal ion, respectively. The numerical value of $30'000 \text{ cm}^{-1}$ relates this scale to the Pauling electronegativity scale where the optical electronegativity of the fluoride ion is arbitrarily chosen to be 3.9. For d electrons the parameter D is approximately equal to $7 B'$ (Lever, 1984) where B' is the Racah parameter (see above). The spin-pairing energy for Co(II) for the ground state is $-2D$ and for the excited state $-2/3D$ (Lever, 1984). Thus, the net change in spin-pairing energy is $4/3D$. ν_{corr} is obtained by subtracting $4/3 D$ from the observed band (ν_{obs})

$$\nu_{\text{corr}} = \nu_{\text{obs}} - 4/3D \quad (6)$$

Based on $B' \approx 580 \text{ cm}^{-1}$, calculated from equation (4) (see above), and the spectral locations ν_{obs} of 350 nm for separate Co(II) tetrathiolate complexes and of 390 nm for the clustered structure (Table II) one obtains for ν_{corr} values of $23'160$ and $20'230 \text{ cm}^{-1}$ for the terminal and bridging thiolate ligands, respectively. Substituting these values and $X_M=1.8$ for Co(II) (Lever, 1984) into equation (5) gives $X_L=2.57$ for terminal thiolate and $X_L=2.47$ for bridging thiolate ligands. The optical electronegativity for the ligands in Co(II)₇-MT is similar to the value of 2.40 previously found for cysteine and methionine sulfur in Co(II)-substituted Azurin (Tennent & McMillin, 1979). Since the spectral parameter B' in Co(II)-MT is not sensitive to cluster formation it can be concluded that the overall red shift

in the charge-transfer region is due entirely to the alteration of the optical electronegativity of the cysteine ligands.

(B) MCD and CD Properties. MCD spectra of Co(II)-MT up to approximately 4 Co(II) equiv exhibit three extrema at (+) 385, (-) 323 and (+) 255 nm and a shoulder near (-) 285 nm (Figure 1-4, bottom). The intensity of the MCD bands increases linearly up to 3 Co(II) equiv. The fourth Co(II) equiv elicits only minor changes in the MCD profile reflecting compensatory changes in the magnetic ellipticity bands. Thus, it appears that MCD in the charge-transfer region provides a sensitive means to distinguish between terminal and bridging thiolate sulfur ligands. Interestingly, the intensity of the positive MCD band at 255 nm is also lowered between 3 and 4 equiv of Co(II). Further addition of Co(II) up to full metal ion occupation displaces the two lowest energy magnetic ellipticity bands to (+) 426 nm and (-) 345 nm. Concomitantly the intensities of the 255 nm band, whose position remains unchanged and of the 345 nm band are markedly lowered. In the titration curve (Figure 1-5, bottom) the red shift of the two lower energy MCD bands associated with increasing Co(II) content manifests itself in a changeover from a positive to a negative slope between 3 and 4 Co(II) equiv. Comparable magneto-chiroptical changes associated with metal-thiolate cluster formation are seen in the titration of apoMT with Fe(II) (see Chapter 3). Similar red shifts of the lowest energy MCD bands but without loss of signal intensity are observed in the clustered structures of Cd(II)- and Hg(II)-substituted MT. The conspicuous reduction of the MCD signals in the Co(II) and Fe(II) containing forms is possibly a feature characteristic of clusters containing paramagnetic metal ions.

Spectral changes somewhat comparable to those observed in the MCD spectra are seen in the CD spectra of Co(II)-MT (Figure 1-4, top). Two oppositely signed CD bands at (-) 366 nm and (+) 316 nm and a positive band at 262 nm emerge upon incorporation of the first 4 Co(II) equiv into apoMT. As in the MCD spectrum the fourth Co(II) equiv elicits relatively smaller changes in the CD profile. Above 4 Co(II) equiv all bands undergo a progressive red shift whereas the amplitudes remain the same or decrease slightly. It is noteworthy that the shoulder discernable near 377 nm in Co(II)₇-MT is well resolved in the Co(II)₄-fragment (see Chapter 2) and in Co(II)₁Zn(II)₆-MT (see below). The close correspondance of the changes in CD with those in MCD and electronic absorption in the charge-transfer region is brought out also by the titration curves in Figure 1-5, where the signals at 390 nm are plotted as a function of metal-to-protein ratio. All plots are characterized by inflection points near 4 and 7 equiv of Co(II).

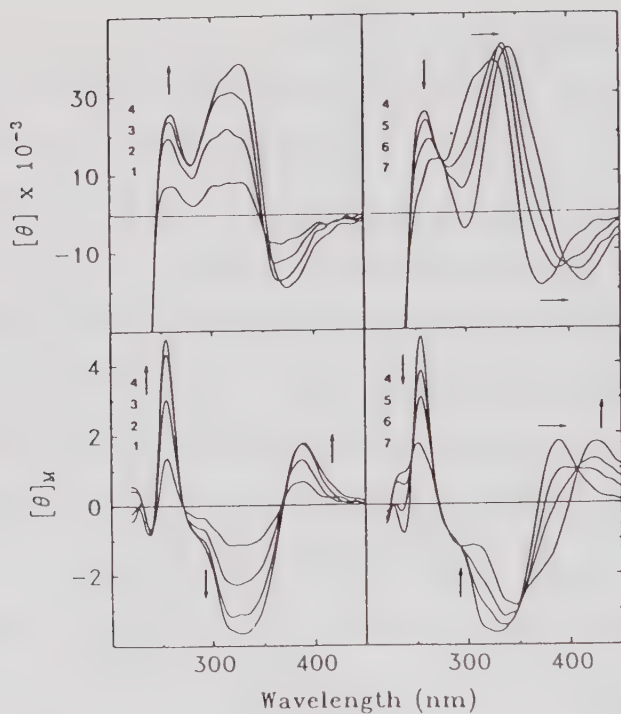


Figure 1-4: CD (top), and MCD (bottom) spectra of Co(II)-MT in the charge-transfer region. The numbers indicate the moles of Co(II) per mole of apoMT and the arrows symbolize the development of the bands with increasing amount of Co(II). $[\theta]$ and $[\theta]_M$ are based on the protein concentration. Conditions are as in Figure 1-1.

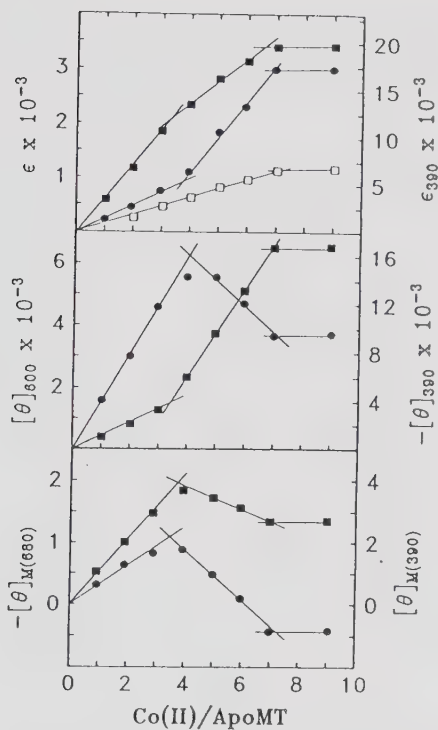


Figure 1-5: Dependency of absorbance (top), CD (middle) and MCD (bottom) on Co(II)-to-apoMT ratio in the d-d (■, □, left scale) and charge-transfer (●, right scale) region. Electronic absorption in the d-d region was measured at 680 nm (■) and 1275 nm (□), respectively.

SPECTROSCOPIC STUDIES OF Co(II)-THIOLATE COMPLEXES EMBEDDED IN DIAMAGNETIC CLUSTERS OF Zn(II)₆-MT and Cd(II)₆-MT

To gain a better understanding of the spectral changes attending the transition of Co(II)-thiolate centers in MT from the nonclustered to the clustered structure fully metal occupied hybride derivatives such as Co(II)₁Zn(II)₆-MT and Co(II)₁Cd(II)₆-MT were prepared. The EPR spectra of Co(II)₁-MT and Co(II)₁Zn(II)₆-MT are shown in Figure 1-6. Both spectra reveal the typical rhombically distorted EPR signals with g-values at $g_x \approx 5.7$, $g_y \approx 4.5$ and $g_z \approx 2.0$ for Co(II)₁-MT, and, $g_x \approx 5.7$, $g_y \approx 3.8$ and $g_z \approx 2.0$ for Co(II)₁Zn(II)₆-MT, respectively (Palmer, 1985). The relative intensities of the EPR signals in both spectra, quantitated by the method of double integration (Palmer, 1967), are similar indicating that no antiferromagnetically coupled vicinal Co(II) ions are present in the mixed metal protein molecule. An EPR spectrum comparable to that of Co(II)₁Zn(II)₆-MT was found also with Co(II)₁Cd(II)₆-MT (spectrum not shown). Thus, these MT-hybrides exhibit cluster structures with terminal and bridging thiolate ligands coordinating Co(II), but they are lacking the antiferromagnetic exchange features Co(II)-thiolate clusters in Co(II)₇-MT. Hence, they are useful to separate the effects of thiolate bridge formation from those resulting from magnetic exchange processes. In the following sections the various spectroscopic properties of Co(II)-thiolate complexes embedded in the diamagnetic clusters of Zn(II)₆-MT and Cd(II)₆-MT are analyzed and compared with those of the nonclustered structure of Co(II)₁-MT and with those of the clustered structure of Co(II)₇-MT (Figure 1-7).

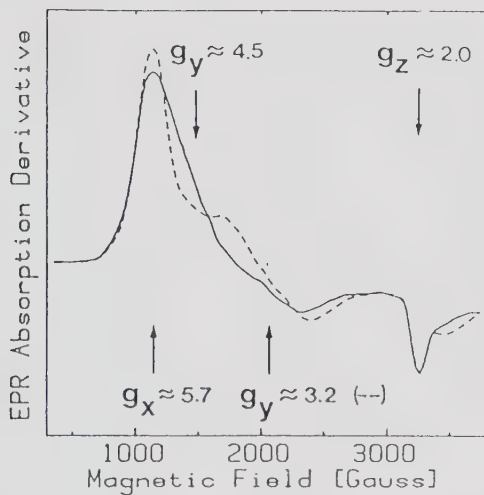


Figure 1-6: EPR spectra of 1.0 mM Co(II)₁-MT (—) and 1.0 mM Co(II)₁Zn(II)₆-MT (---) in 160 mM Tris-HCl, pH 8.3. Conditions: microwave power 0.125 mW, microwave frequency 9.239 GHz, 3.4 K.

(A) Electronic Absorption Properties. Figure 1-7 (top, right) shows the electronic absorption spectra in the d-d region of the clustered structures of $\text{Co(II)}_7\text{-MT}$, $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ and $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$, as well as of the nonclustered structure of $\text{Co(II)}_1\text{-MT}$. Overall, in the three clustered structures Co(II) displays nearly the same absorption profile. There are only small differences in position and intensity of the bands. Thus, it may be concluded that the coordination geometry of the Co(II) embedded in the diamagnetic metal thiolate matrix resembles that of $\text{Co(II)}_7\text{-MT}$. The energy difference between the corresponding spin orbit coupling components of 1300 and 2535 cm^{-1} in $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ and 1410 and 2245 cm^{-1} in $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$, which is slightly larger than that of $\text{Co(II)}_7\text{-MT}$ (see above), can be attributed to an increased distortion of the Co(II) -site in the Zn(II) - and Cd(II) -matrix, respectively.

In the charge-transfer region, too, the locations of the band maxima at 315 and at 380 (sh) nm in $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ and 305 (sh) and 380 (sh) nm in $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$ are similar to those of $\text{Co(II)}_7\text{-MT}$ (see above) and are quite different from those of $\text{Co(II)}_1\text{-MT}$ at 305 and 350 nm (sh) (Figure 1-7 top, left). This correspondance unambiguously demonstrates that the red shift of the Co(II) -thiolate bands upon cluster formation is due solely to the emergence of bridging thiolate ligands. While the absorption profiles of $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ and $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$ are comparable, $\text{Co(II)}_7\text{-MT}$ differs significantly in intensity above 320 nm. The first charge-transfer band near 390 nm is about 50% more intense in $\text{Co(II)}_7\text{-MT}$ (Table II). In contrast to this wide variation, all complexes examined display at 315 nm nearly a constant Co(II) -thiolate absorbancy of about $1150\text{ M}^{-1}\text{ cm}^{-1}$ per sulfur atom.

(B) MCD Properties. The most conspicuous feature of the d-d region is that the MCD spectra of $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ and $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$ and $\text{Co(II)}_7\text{-MT}$ display quite comparable band envelopes with the first two negative bands located at similar positions (Figure 1-7 bottom, right). However, the amplitudes of these magnetic ellipticity bands differ substantially. $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ and $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$ have the largest $[\Theta]_{\text{M}}$ -values (Table II). The two low energy MCD bands of $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ exceed those of $\text{Co(II)}_1\text{-MT}$ by 47% (748 nm) and 55% (680 nm), respectively. An analogous comparison of $\text{Co(II)}_7\text{-MT}$ with $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$ yields 20% (752 nm) and 73% (690 nm) larger magnetic ellipticities in the latter. Note that almost no differences in intensity among the electronic absorption spectra exist. Since the strong MCD features of Co(II) -complexes are conditioned largely by the paramagnetism of Co(II) (C-term) (Johnson et al., 1982) it is conceivable that the lower intensity of some of the magnetic ellipticity bands in $\text{Co(II)}_7\text{-MT}$ is related to the loss of paramagnetism brought about by antiferromagnetic coupling (Vasak & Kägi, 1981) and hence, to a partial loss of C-term contribution. Principally, a C-term would not be expected for tetrahedrally coordinated Co(II) because of its nondegenerate $^4\text{A}_2$ ground state. However, because of level mixing by spin orbit coupling this argument is no longer applicable to the $\nu_3[{}^4\text{A}_2 \rightarrow {}^4\text{T}_1(\text{P})]$ transition. Owing to spin orbit coupling the ground state is split and consequently a C-term is obtained. This was documented by Kato and Akimoto (1974) who have analyzed the MCD spectra of several inorganic tetrahedrally coordinated Co(II) -complexes and concluded that the MCD bands of the ν_3 -transition arise predominantly from a C-term. Importantly, these authors pointed out that the main features of the MCD profile arise

from spin orbit splitting of the upper state $^4T_1(P)$. Since the spin orbit coupling components in the ν_3 -transition differ appreciable in the species examined in this work (see above) it is probable that these differences also underlie the alterations in shape of the ν_3 -MCD bands when the clustered structure is formed.

In contrast to the appreciable intensity differences in the d-d region, the MCD profiles of $\text{Co(II)}_7\text{-MT}$ and of both matrix species are very comparable in the low energy position of the charge-transfer region (Figure 1-7 bottom, left). Thus, this data show convincingly that the pronounced red shift of the MCD signal reflects the transformation of some terminal sulfur ligands to bridging ligands. At the high energy side of the charge-transfer region the magnetic ellipticities of the overall MCD profiles are slightly larger in $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ and $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$ than in $\text{Co(II)}_7\text{-MT}$. As mentioned with respect to the MCD signals in the d-d region this intensity deficit in the MCD amplitudes in $\text{Co(II)}_7\text{-MT}$ could be due to partial loss of C-term.

(C) CD Properties. Interestingly, indications for the characteristic band splitting in the d-d region of the first low energy CD band in $\text{Co(II)}\text{-MT}$ above 4 Co(II) equiv can also be noticed in the matrix spectra of $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ and $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$. The signs of the 768 nm CD band in all 3 Co(II) -complexes having a clustered structure are the same and are opposite to that of $\text{Co(II)}_7\text{-MT}$. Thus, the splitting of this d-d band must be a property of the clustered structure. While the magnitude of the first low energy band in $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$ is comparable to that of $\text{Co(II)}_7\text{-MT}$ the ellipticity of this band in $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ is about 3.5 times as intense as in $\text{Co(II)}_7\text{-MT}$ (Figure 1-7 middle, right).

In the charge-transfer region, the CD features of $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ and $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$ are also similar to those found for $\text{Co(II)}_7\text{-MT}$. However, in the two former species a new positive CD maximum appears at 372 and 370 nm, respectively (Figure 1-5 middle, left). $\text{Co(II)}_7\text{-MT}$ displays at this position a shoulder. The inflection points between this new band and the CD band above 400 nm in $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ and $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$ are located approximately at 390 nm and are thus coinciding with the position of the first charge-transfer band in the corresponding absorption spectra. The association of such oppositely signed CD bands with a single transition can be taken as evidence for excitonic coupling of juxtaposed identical electronic transition dipole moments within a composite chromophoric system. Recently, it has been demonstrated theoretically that such interactions play a major role in bringing about the chiroptical features of $\text{Cd(II)}_7\text{-MT}$ (Willner, 1987). In this derivative the typical biphasic CD signal arises from bridging thiolate ligands assembled in the clustered structure. Comparable chiroptical features occur also in Hg(II)-MT (Bernhard, 1986). Thus, it is likely that such interactions are also present in Co(II) -substituted MT's.

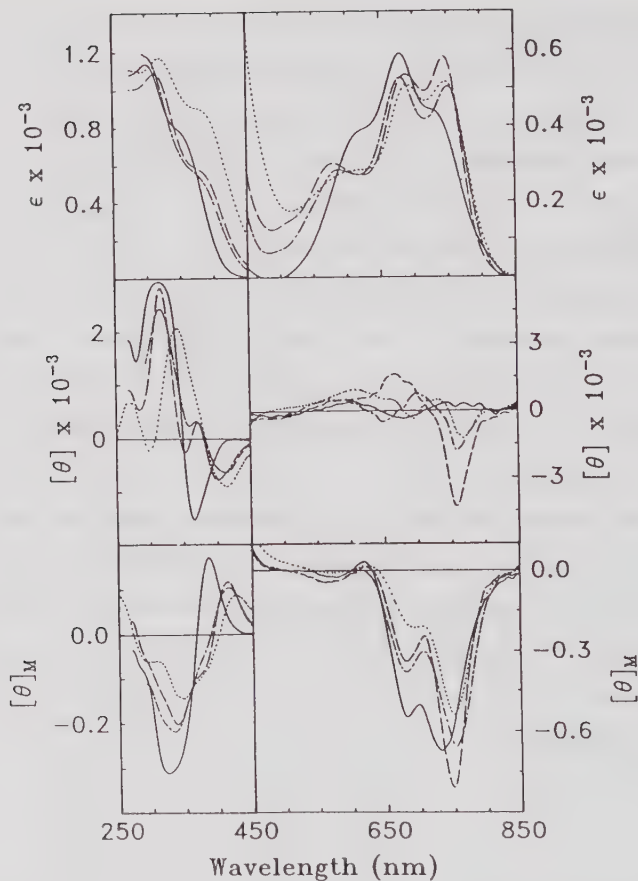


Figure 1-7: Electronic absorption (top), CD (middle), and MCD (bottom) spectra of $\text{Co(II)}_1\text{-MT}$ (—), $\text{Co(II)}_7\text{-MT}$ (·····), $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ (---), and $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$ (- - -). ϵ , $[\theta]$ and $[\theta]_M$ are calculated per cobalt atom in the d-d (right) and per sulfur atom in the charge-transfer (left) region. Conditions: 160 mM Tris-HCl, pH 8.3 at room temperature.

Table II Spectral Data of tetrahedral tetrathiolate Co(II)-Complexes in MT

Complex	$\nu_3 [A_2(F) \rightarrow {}^4T_2(P)]^a$		$S^{\cdot-} \rightarrow Co(II)$ charge transfer ^b	
Co ₁ -MT	abs ^c	730,sh (0.46)	680 (0.63)	620,sh (0.40)
	CD ^d	770 -	680 (-0.37)	606 (0.42)
	MCD ^e	730 (-0.67)	680 (-0.55)	616 (0.03)
Co ₇ -MT	abs	748 (0.51)	690 (0.51)	600,sh (0.29)
	CD	768 (-1.25)	732 (0.53)	695 (-0.33)
	MCD	750 (-0.55)	688 (-0.22)	616 (0.01)
Co ₁ 2n ₆ -MT	abs	746 (0.60)	680 (0.54)	580 (0.29)
	CD	757 (-4.30)	667 (1.62)	
	MCD	748 (-0.81)	660 (-0.34)	616 (0.01)
Co ₁ CD ₆ -MT	abs	752 (0.51)	680 (0.53)	590 (0.29)
	CD	760 (-1.78)	700 (0.78)	650 (-0.51)
	MCD	752 (-0.66)	680 (-0.38)	616 (0.02)
Co ₁ 2n ₆ -MT	abs	746 (0.60)	680 (0.54)	580 (0.29)
	CD	757 (-4.30)	667 (1.62)	
	MCD	748 (-0.81)	660 (-0.34)	616 (0.01)
Co ₁ CD ₆ -MT	abs	752 (0.51)	680 (0.53)	590 (0.29)
	CD	760 (-1.78)	700 (0.78)	650 (-0.51)
	MCD	752 (-0.66)	680 (-0.38)	616 (0.02)

a) Values are calculated per cobalt atom

b) Values are calculated per sulfur atom

c) In nm ($\epsilon \times 10^{-3}$, M⁻¹ cm⁻¹)d) In nm ($[\theta] \times 10^{-3}$, deg cm² dmol⁻¹)e) In nm ($[\theta]_M$, deg cm² dmol⁻¹ Gauss⁻¹)

1-3 Conclusion

The spectroscopic results presented in this chapter document primarily the usefulness of MCD spectroscopy in distinguishing terminal and bridging thiolate sulfur ligands of tetrahedral Co(II)-complexes in Co(II)-MT. Differentiating MCD features occur both in the d-d as well as in the charge-transfer region. There are significant changes in shape and position of the MCD bands, as well as in their intensities when in cluster formation terminal thiolate ligands are transformed into bridging ligands. The highly characteristic alterations of the two large negative MCD bands in the d-d region at 680 and 730 nm are probably caused by changes in spin orbit coupling of the d-electrons in Co(II) when the tetrahedral coordination geometry is distorted by the framework of the cluster. Antiferromagnetic exchange between the paramagnetic centers in Co(II)₇-MT does not seem to affect the position and shape of these MCD bands although it may have some influence on their intensities. To document such effects temperature dependent studies over a wide range of temperature would be required. Since paramagnetic chromophores give temperature dependent MCD spectra, whereas diamagnetic centers are temperature independent, it is expected that the MCD spectra of Co(II)₁-MT, Co(II)₁Zn(II)₆-MT and Co(II)₁Cd(II)₆-MT are more sensitive against variations of temperature than Co(II)₇-MT where the Co(II) centers are antiferromagnetically coupled. The most conspicuous feature in the charge-transfer region is the red shift of the lowest energy MCD bands upon transformation of terminal into bridging thiolate ligands. There is also a loss of intensity of the MCD bands upon cluster formation. Of the three clustered structures Co(II)₇-MT displays the

lowest amplitudes but the differences are not as pronounced as in the d-d region. In the CD spectra the distinguishing feature of cluster formation are the splitting of the 750 nm band and the red shift of the lowest energy CD band in the charge-transfer region. In the electronic absorption spectra the emergence of the cluster is manifested by the 20-30 nm shift of the charge-transfer absorption edge. This marked shift appears to be the result of a drop in optical electronegativity upon twofold metal coordination to thiolate sulfur, i.e. transformation of a terminal into a bridging sulfur ligand. The higher energy bands in the charge-transfer absorption profile are somewhat less affected by cluster formation. The molar absorbance of the 315 nm band affords a convenient measure of the number of thiolate ligands involved in metal binding. Cluster formation is also without effect on position, shape and intensity of the electronic absorption in the near-infrared region. The ligand field parameters Δ_t and B' do not discriminate between terminal and bridging thiolate sulfur.

C H A P T E R 2

SPECTROSCOPIC PROPERTIES OF THE α -FRAGMENT OF METALLOTHIONEIN SUBSTITUTED WITH COBALT(II) AND CADMIUM(II)

2-1 Introduction

Although spectroscopic information on the structural characteristics of MT is now substantial it reflects in most cases the sum of the unresolved contributions of both clusters present in the native protein. The only two spectroscopic studies on record dealing with isolated clusters are the ^{113}Cd -NMR measurements of the α -fragment from rat liver MT which revealed four ^{113}Cd resonances with chemical shifts comparable to those found in $\text{Cd}_7\text{-MT}$ (Boulanger et al., 1982) and the CD and MCD measurements of the cadmium containing form (Zelazowski et al., 1984). This chapter explores in more detail the structural properties of the α -fragment and compares them with the corresponding features of the uncleaved protein. For this purpose the α -fragment of rabbit-liver MT-1 was isolated and the spectroscopic properties of its Co(II) derivative examined. In this study the coordination geometry of the metal binding sites of the C-terminal cluster and its mode of formation is explored by electronic absorption, MCD, CD and EPR techniques. These results provide evidence for a T_d -type symmetry in the binding of the first three Co(II) ions. The different spectroscopic features associated with the binding of the fourth Co(II) ion imply a different coordination geometry for this metal binding

site. Independent support for the nonidentity between the α -fragment and the uncleaved protein comes from its Cd(II) derivative. Hence, electronic absorption and CD features of the Cd(II)-substituted α -fragment are also discussed.

2-2 Results and Discussion

The ability of the C-terminal fragment of rat liver MT to bind four diamagnetic Cd(II) ions was demonstrated by Winge and Miklossy (1982). In the present study the same metal-to-protein stoichiometry was confirmed also in the isolated α -fragment of rabbit liver MT. The amino acid composition of this isolated α -fragment is shown in Table III. By the replacement of diamagnetic Cd(II) with paramagnetic Co(II) a Co(II)₄-fragment was obtained (see below) and subjected to detailed spectroscopic studies. The first part of this chapter presents electronic absorption, CD and MCD spectra in the visible and high-energy region of the fully metal occupied Co(II)₄-fragment and compares them with those of Co(II)₇-MT (dashed lines in Figure 2-1 and 2-2). The second part deals with the formation of the tetranuclear cluster as monitored by electronic absorption, MCD and EPR measurements. A third part discusses the unusual differences in CD titration with Cd(II) ions of the α -fragment.

Table III Amino Acid Composition of α -Fragment of Rabbit
Liver Metallothionein 1

Residue	Analysis ^{a)}
Cys ^{b)}	11.11 (11)
Asx	1.13 (1)
Thr	1.06 (1)
Ser	3.74 (4)
Glx	1.15 (1)
Pro	0.72 (1)
Gly	3.49 (3)
Ala	3.77 (4)
Ile	0.89 (1)
Lys	3.93 (4)
Total residues	31

a) The amino acid analysis was performed after performic acid oxidation
(16 hrs at 0° C) and hydrolysis (20 hrs at 110° C) in 6 N HCl.

b) analyzed as cysteic acid.

SPECTRAL PROPERTIES OF Co(II)_4 -FRAGMENT

(A) **Visible Region of Co(II)_4 -Fragment.** Figure 2-1 shows the electronic absorption, MCD and CD spectrum of the fully metal occupied Co(II)_4 -fragment (solid lines) and Co(II)_7 -MT (dashed lines) under the same experimental conditions. The electronic absorption spectrum (Figure 2-1, top) of the Co(II)_4 -fragment reveals two bands with maxima at 738 and 685 nm and a shoulder near 620 nm with molar absorptivities of 350, 390 and $280 \text{ M}^{-1}\text{cm}^{-1}$, respectively. These features are typical of tetrahedral Co(II) -thiolate coordination (see Chapter 1) and closely resemble those reported for inorganic tetrathiolate Co(II) -complexes (Anglin & Davison, 1975; Holah & Coucouvanis, 1975; Lane et al., 1977; Swenson et al., 1978; Dance, 1979) and for Co(II) substituted metalloproteins with four cysteine ligands, for example, Co(II) -rubredoxin (May & Kuo, 1978) and horse liver alcohol dehydrogenase with Co(II) bound at the noncatalytic metal binding site (Maret et al., 1979). Interestingly, the molar absorptivity per cobalt are about one third lower in the Co(II)_4 -fragment as compared with Co(II)_7 -MT (Table IV). The resolved d-d band pattern can be assigned to the spin allowed and symmetry-forbidden $\nu_3[{}^4\text{A}_2 \rightarrow {}^4\text{T}_1(\text{P})]$ transition split by spin orbit coupling (for details see Chapter 1). In the absorption spectrum of the Co(II)_4 -fragment only 3 spin orbit coupling components with an energy separation of 1050 and 1530 cm^{-1} are clearly resolved (Figure 2-1, top). This energy separation is smaller than that obtained with fully metal occupied rabbit liver Co(II)_7 -MT of 1146 cm^{-1} and 2175 cm^{-1} (see Chapter 1) and is larger than that to be expected from spin orbit coupling alone (Cotton et al., 1961). The presence of the $\nu_2[{}^4\text{A}_2 \rightarrow {}^4\text{T}_1(\text{F})]$ transition in the

near-infrared region (Figure 2-4) at 1275 nm offers additional proof for tetrahedral coordination of at least some Co(II) ions in the fragment (see below).

Evidence for tetrahedral coordination is provided also by MCD measurements (Figure 2-1, middle). The ν_3 -transition of the Co(II)_4 -fragment in the visible region between 850 and 450 nm displays a strong negative MCD band at 743 nm with a pronounced shoulder near 690 nm and small positive and negative MCD bands at 617 and 573 nm, respectively. The positions of the first three low energy MCD bands coincide with those of the resolved spin orbit coupling components in the electronic absorption spectrum (Figure 2-1, top). Comparison of this MCD profile with those of well defined Co(II)-complexes of different coordination number and geometry (Kaden et al., 1974) denotes the existence of T_d -type symmetry at the metal binding site of the fragment. Similar MCD features have also been found in a number of tetrahedral and pseudotetrahedral Co(II)-complexes (Kato & Akimoto, 1974). The MCD features arise predominantly from the temperature dependent C-term (see Chapter 1). The molar magnetic ellipticity $[\theta]_M$ at 743 nm, when calculated per Co(II) ion bound, yields a value which is about 40% lower in the case of the Co(II)_4 -fragment than that found with Co(II)_7 -MT (Table IV). A similar decrease in intensity was also found in the corresponding absorption band (see above). The possible cause for this intensity drop is discussed in the second part of this chapter (see below).

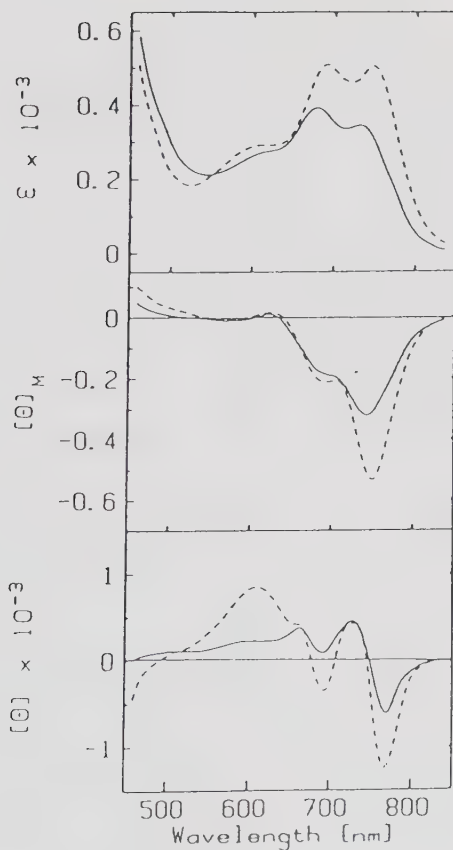


Figure 2-1: Absorption (top), MCD (middle) and CD (bottom) spectra of 0.17 mM Co(II)_4 -fragment (solid lines) and 0.17 mM Co(II)_7 -MT (dashed lines) in 160 mM Tris-HCl, pH 8.3, at room temperature. ϵ , $[\Theta]_M$ and $[\Theta]$ are based on the concentration of Co(II). (For details see Experimental Procedures)

The low energy CD spectrum of the Co(II)_4 -fragment (Figure 2-1, below) displays a negative band at 768 nm and three positive bands at 728 , 663 and near 600 nm with molar ellipticities $[\Theta]$ of 2860, 1860, 1570 and 1050 $\text{deg cm}^2 \text{ dmol}^{-1}$, respectively. The CD spectrum, in marked contrast to the MCD spectrum, is very sensitive to conformational and vicinal changes at the metal binding site and, hence, provides less unambiguous information about the coordination geometry (Vallee and Holmquist, 1980). Again the CD-bands of the Co(II)_4 -fragment differ quite appreciably from those of Co(II)_7 -MT. The CD bands associated with the d-d transition of the fragment are about half as intense as those of the holoprotein.

(B) High-Energy Region of Co(II)_4 -Fragment. Since apoMT does not absorb appreciably above 250 nm (Vasak & Kägi, 1983) all spectral features of the Co(II) -substituted fragment must arise from metal binding. Thus, the electronic absorption features (Figure 2-2, top) below the d-d region must be attributed either to charge-transfer transitions or to ligand internal excitations (Mastropolo et al., 1977) affected by the interaction with the metal ion. The electronic absorption spectrum of the fully metal occupied fragment displays a shoulder near 390 nm and a maximum at 320 nm with molar extinction coefficient of $1925 \text{ M}^{-1} \text{ cm}^{-1}$ and $3180 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. The 320 nm absorption band with a molar absorptivity of about $1150 \text{ M}^{-1} \text{ cm}^{-1}$ per thiolate ligand was also seen in Co(II) -substituted forms of rubredoxin (May & Kuo, 1978), horse liver alcohol dehydrogenase (Drum & Vallee, 1970; Maret et al., 1979) and model complexes (Lane et al., 1979). A comparison of the Co(II) -based molar absorptivities of the Co(II)_4 -fragment in the charge-transfer region with those of Co(II)_7 -MT

reveals that in contrast to the d-d and near infrared region, where marked differences exist (see above), the charge-transfer regions are almost identical. A higher resolution of this spectral region is provided by the corresponding MCD and CD spectra (Figure 2-2, middle and bottom). The MCD spectrum of the high energy region displays MCD bands of opposite sign at (+) 427 and (-) 347 nm, and a negative shoulder near (-) 290 nm and a maximum at (+) 255 nm. The MCD bands of the Co(II)_4 -fragment and Co(II)_7 -MT are at the same positions but differ somewhat in signal size. The CD spectrum of the Co(II)_4 -fragment displays still higher resolution than the corresponding MCD spectrum and is characterized by a number of ellipticity bands with extrema at (-) 415, (+) 375, (+) 324 and (+) 255 nm (Table IV). The resolved CD profile of the Co(II)_4 -fragment differs appreciable from Co(II)_7 -MT and has markedly lower amplitudes per Co(II) . A possible explanation for this effect is the increased symmetry of the Co(II) sites in the Co(II)_4 -fragment suggested also by the decreased energy separation of the spin orbit coupling components of the ν_3 -transition (see above). On the basis of a comparison of the tetrahedrally coordinated Co(II)_7^- and Ni(II)_7 -MT derivatives, all three low energy CD bands in both metal derivatives were assigned to $S \rightarrow \text{Me(II)}$ excitations (Vasak et al., 1981). Hence, it is inferred that the same assignment holds also for the first three low-energy CD bands in the Co(II)_4 -fragment.

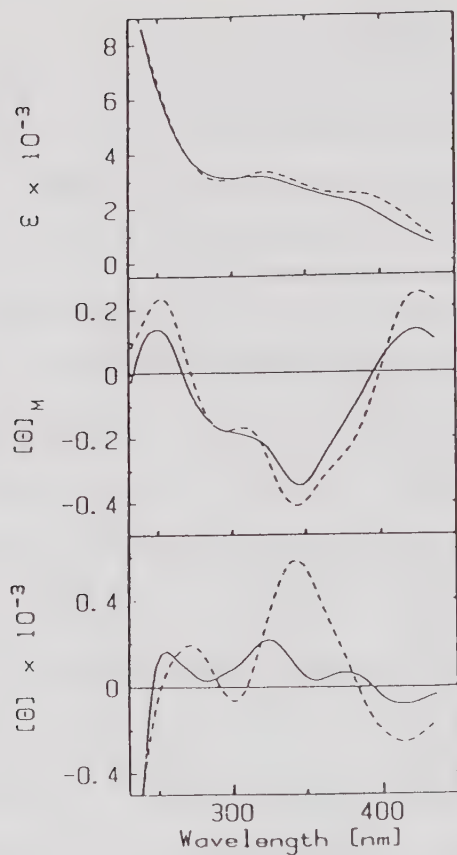


Figure 2-2: Absorption (top), MCD (middle) and CD (bottom) spectra of the Co(II)₄-fragment (solid lines) and Co(II)₇-MT (dashed lines). ϵ , $[\Theta]_M$ and $[\Theta]$ are based on the concentration of Co(II). Conditions are as in Figure 2-1.

Table IV Comparison of Electronic Absorption, MCD, and CD Maxima of Fully Metal Occupied Co(II)_4 -Fragment and Co(II)_7 -Metallothionein (in Parentheses)

Absorption		MCD		CD	
[nm]	$\epsilon \times 10^{-3} \text{ a)}$	[nm]	$[\theta]_M \text{ a)}$	[nm]	$[\theta] \times 10^{-3} \text{ a)}$
1272 (1275)	0.11 (0.16)	743 (750)	-0.330 (-0.550)	768 (768)	-0.715 (-1.247)
738 (748)	0.35 (0.51)			728 (732)	0.465 (0.530)
-		sh585 (688)	-0.192 (-0.222)	690 (695)	0.044 (-0.332)
595 (690)	0.39 (0.51)	617 (616)	0.006 (0.004)	663 (sh662)	0.393 (0.477)
-		573 (572)	-0.001 (-0.021)	sh600 (610)	0.262 (0.924)
sh610 (sh600)	0.28 (0.29)			500 (-)	0.103 (-)
-		427 (426)	0.127 (0.250)	415 (417)	-0.800 (-2.615)
sh390 (sh390)	2.14 (2.44)	347 (345)	-0.332 (-0.414)	375 (-)	0.670 (-)
320 (320)	3.18 (3.33)	sh290 (sh290)	0.179 (0.174)	324 (343)	2.400 (5.928)
		255 (255)	0.095 (0.261)	255 (275)	2.000 (2.040)

a) Values are calculated per cobalt atom.

CLUSTER FORMATION IN Co(II)-FRAGMENT

To gain insight into the pathway of metal incorporation and cluster formation the changes in the electronic absorption, MCD and EPR spectra were monitored upon incremental additions of Co(II) to the apofragment.

(A) Electronic Absorption Properties. The electronic absorption features emerging in the charge-transfer region are shown in Figure 2-3 (left). In spite of the apparent similarity of the various profiles there are distinct changes attending the increase in metal occupancy. Upon addition of the first 2 Co(II) equiv to the apofragment the absorption envelope reveals two rather well resolved charge-transfer maxima at 305 and 350 nm. The subsequent addition of the remaining 2 Co(II) equiv results in a bathochromic shift of these maxima to 320 and 390 nm, respectively. Further addition of Co(II) ions to the Co(II)_4 -fragment does not affect the spectrum. Differences comparable to those observed between the partially and fully metal saturated Co(II)-fragment have also been observed by Dance (1979) in the spectra of crystallographically defined tetrahedral thiolate complexes of mononuclear and tetranuclear Co(II)-benzenethiolate, i.e., $[\text{Co}(\text{SPh})_4]^{2-}$ and $[\text{Co}_4(\text{SPh})_{10}]^{2-}$, respectively. While the former complex contains only terminal sulfur ligands, the latter complex is made up of four terminal and six bridging sulfurs. Thus, the red shift in the absorption spectrum of the Co(II)_4 -fragment attending the filling up process must be due to the involvement of bridging thiolate sulfur in metal coordination and reflects probably the stronger polarization of the bridging thiolate ligands by the two adjacent metal ions. The participation of thiolate ligands in all

complexes formed in titration with Co(II) is documented by an increase of the molar absorptivity followed at 315 nm as a function of increasing metal binding site occupation (Figure 2-3, inset). The change of the slope at about 1.7 Co(II) equiv from 4330 to 2150 reflects the transition from mononuclear complexes containing solely terminal thiolates to oligonuclear entities where the metals are connected by bridging thiolate ligands. Accepting that the molar extinction coefficient for all 11 Co(II)-coordinated thiolate sulfurs of the α -fragment is comparable ($\epsilon_{315} \approx 1150 \text{ M}^{-1} \text{ cm}^{-1}$; see Chapter 1), it follows from the initial slope that the first 1.7 Co(II) equiv must be coordinated by four terminal thiolate ligands. More direct evidence for the initial formation of such isolated T_d -sites is provided also by EPR titration (see Figure 2-6, right). The decrease in slope setting in above 1.7 Co(II) equiv signals partial sharing of the ligands leading to a reduction in Co(II)-to-thiolate stoichiometry. Thus, on the basis of the same molar absorbancy for the thiolate sulfurs, binding of 3 Co(II) equiv to apofragment require only nine ligands for metal coordination (see Figure 2-3, inset).

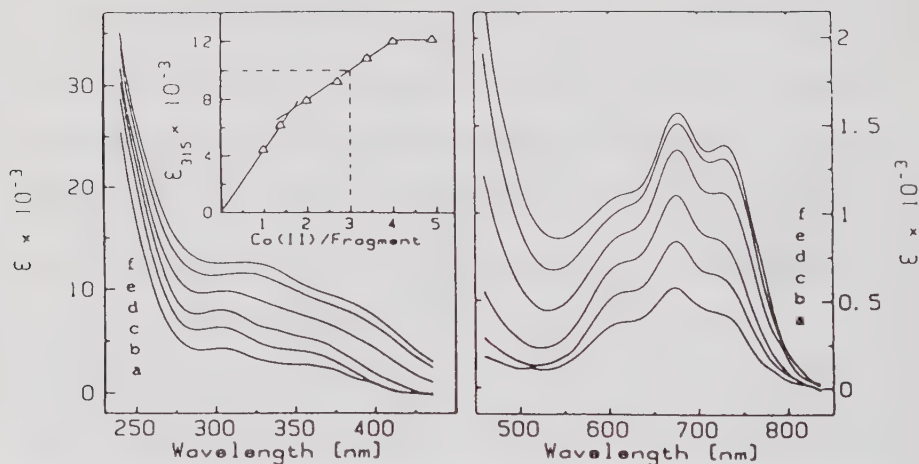


Figure 2-3: (Left) Absorption spectra in the charge-transfer region of the Co(II)-fragment as a function of Co(II)-to-protein ratio. The moles of Co(II) per mole of apofragment are: a, 1.0; b, 1.5; c, 2.1; d, 2.7; e, 3.4; f, 4.0. (Inset, left) Dependency of the absorbance at 315 nm on Co(II)-to-protein ratio. (Right) Absorption spectra in the d-d region of the Co(II)-fragment as a function of Co(II)-to-protein ratio. The moles of Co(II) per mole of apofragment are: a, 1.0; b, 1.5; c, 2.1; d, 2.7; e, 3.4; f, 4.0. ϵ is based on the protein concentration. Conditions are as in Figure 2-1.

The effect of increasing Co(II)-to-apofragment ratios on the absorption spectrum in the visible region is shown in Figure 2-3 (right). The absorption features of the Co(II)-fragment occupied incompletely by Co(II) resemble those of the fully metal occupied Co(II)_4 -fragment and are typical of a tetrahedral tetrathiolate coordination. The same conclusion can be drawn from the corresponding MCD and EPR spectra (Figure 2-5 and 2-6).

However, there are also some distinct spectral changes developing with the filling up of the metal binding sites by Co(II). Up to binding of about 2 equiv of Co(II) the d-d profile increases in intensity without changing shape. Addition of the remaining 2 Co(II) equiv to the apofragment alters the positions of the spin orbit coupling components of the ν_3 -transition. This is reflected in the shifts from 630, 680 and 725 nm to 620, 685 and 738 nm, respectively, resulting in increased energy separation between the corresponding spin orbit coupling components from 1170 cm^{-1} and 910 cm^{-1} to 1530 cm^{-1} and 1050 cm^{-1} , respectively. It is also noteworthy that the amplitudes of the 685 and 738 nm do not change proportionately to the number of Co(II) equiv incorporated. This is in contrast to the nearly linear increase in intensity observed in the reconstitution of Co(II)-MT (see Chapter 1) and is taken as an indication of a difference in the mode of Co(II) binding to the fragment.

To get more quantitative information on this latter aspect the stepwise incorporation of Co(II) ions was studied also by monitoring the electronic absorption changes in the near-infrared region (Figure 2-4). Measuring the absorbance at 1275 nm a linear increase is observed up to 3 equiv of Co(II) added (Figure 2-4, right). Addition of the fourth Co(II) equiv produces no further change in absorbance at this spectral position. However, a slight increase in absorption is discerned at the high energy edge of the spectral envelope. The difference between the last two titration steps yields a difference spectrum with a maximum near 1100 nm and a difference molar extinction coefficient of about $35\text{ M}^{-1}\text{ cm}^{-1}$ per Co(II) ion (Figure 2-4, inset). The about five-times lower absorption increment upon addition of the last Co(II) is suggestive of its incorporation into a metal binding site of nontetrahedral symmetry.

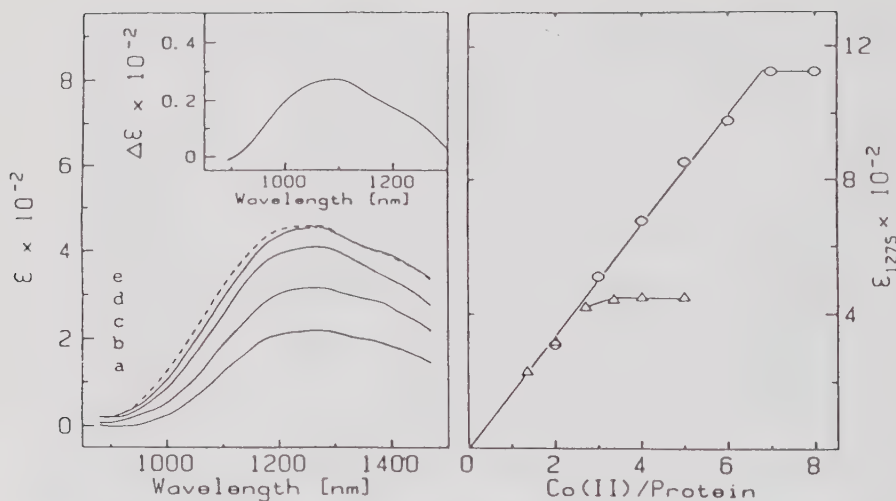


Figure 2-4: (Left) Absorption spectra in the near infrared region of the Co(II)-fragment (0.17 mM) in D₂O (160 mM Tris-DCl, pH 8.3) as a function of Co(II)-to-protein ratio. The moles of Co(II) per mole of apofragment are: a, 1.3; b, 2.0; c, 2.7; d, 3.4; e (dashed line) 4.0.

(Inset, left) Difference absorbance spectrum of spectra d vs. e. (Right) Dependency of absorbance at 1275 nm as a function of Co(II)-to-protein ratio of the Co(II)-fragment (Δ) and of Co(II)-MT ($\circ$). ϵ is based on the protein concentration.

(B) MCD Properties. Further support for a different metal binding site in the Co(II)-fragment comes from MCD studies (Figure 2-5). The MCD profiles resulting from the addition of the first three Co(II) resemble that of the fully metal saturated Co(II)₄-fragment (Figure 2-5). On the basis of a qualitative and quantitative comparison between the MCD profiles of Co(II) substituted proteins and those of Co(II) model complexes the coordination geometry can be deduced (Vallee & Holmquist, 1980). Thus, the MCD spectra generated by the addition of the first 3 equiv of Co(II) ions are typical of tetrahedral metal coordination. Pentacoordination would be characterized by the presence of two rather strongly structured bands in MCD as well as in absorption. In contrast, the intensity of absorption spectra of octahedral complexes (e.g. [Co(H₂O)₆]²⁺) is generally low, $\epsilon=4-10 \text{ M}^{-1} \text{ cm}^{-1}$ and their MCD spectra are characterized by a slightly structured negative band centered at higher energy with a magnetic molar ellipticity $[\Theta]_M$ of -0.006 to $-0.028 \text{ deg cm}^2 \text{ dmol}^{-1} \text{ G}^{-1}$. Thus, the failure of the last Co(II) to elicit in the d-d region further increments in absorption and MCD is compatible with its binding in an octahedral environment. For the latter coordination geometry additional ligands would be required. The possibility of water coordination to the fourth metal binding site was probed by studying the effect of I⁻ ions on the electronic absorption profile up to a 35 molar excess of I⁻ over Co(II). However, no changes in the d-d as well as charge-transfer profiles were discerned, implying that the postulated additional ligands must be contributed also by the protein molecule.

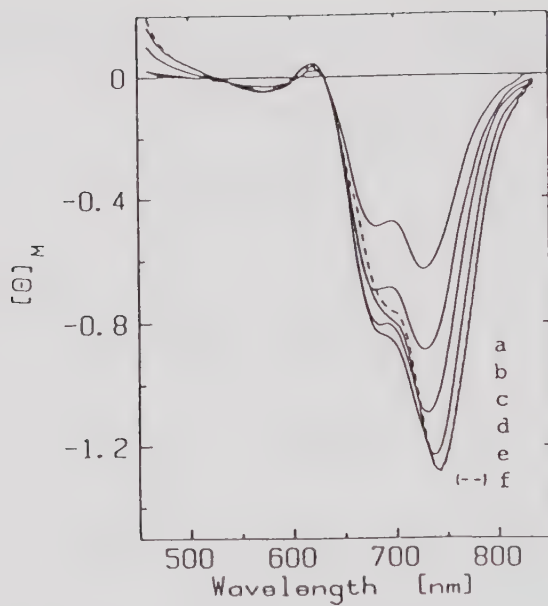


Figure 2-5: MCD spectra in the d-d region of the Co(II)-fragment as a function of metal-to-apofragment stoichiometry. The moles of Co(II) per mole of apofragment are: a, 1.0; b, 1.5; c, 2.1; d, 2.7; e, 3.4; f, 4.0. $[\Theta]_M$ is based on the protein concentration. Conditions are as in Figure 2-1.

(C) EPR Properties. The building up of the cluster was further studied using low temperature (3.4 K) EPR measurements. The EPR spectra recorded at varying ratios of Co(II)-to-apofragment display qualitatively the same profiles but differ markedly in intensity. The spectrum of a sample with a metal-to-apofragment stoichiometry of 2.1 is shown in Figure 2-6 (left). It is typical of rhombically distorted high-spin Co(II) complexes with g-values of $g_x \approx 5.7$, $g_y \approx 4.5$ and $g_z \approx 2.0$. Up to the binding of about 1.7 Co(II) equiv the intensity of the signal at $g_x \approx 5.7$ increases linearly with Co(II) addition. Beyond this ratio the intensity declines again reaching a minimum at about 3 Co(II) equiv. This effect is attributable to antiferromagnetic coupling of vicinal tetrahedral tetrathiolate Co(II)-complexes sharing a common thiolate ligand and signals the emergence of the clustered structure in the fragment. The intensity drop in the EPR spectra parallels the red shift of the electronic spectra in the charge-transfer region which was attributed previously to the participation of bridging thiolates in metal binding (Vasak & Kägi, 1981). Reappearance of an EPR signal with comparable g-values upon the addition of the last equiv of Co(II) may be related to its somewhat different mode of binding. As suggested above this Co(II) may be linked nontetrahedrally through bridging thiolate ligands to the nearly EPR silent Co(II)_3 -cluster and may reflect a perturbation of the antiferromagnetic exchange interactions within the Co(II)_4 -fragment. Thus, the partial recovery of the EPR signal upon the addition of the fourth Co(II), which is not seen in Co(II)_7 -MT, is another indication of the nonidentity of the cluster structure in the holoprotein and the fragment. A similar reappearance of the amplitude of the EPR signal was observed for *Neurospora crassa* MT when in addition to

the 2 Co(II) equiv, coordinated in a binuclear tetrahedral tetrathiolate cluster a third Co(II) was bound (Beltramini et al., 1984).

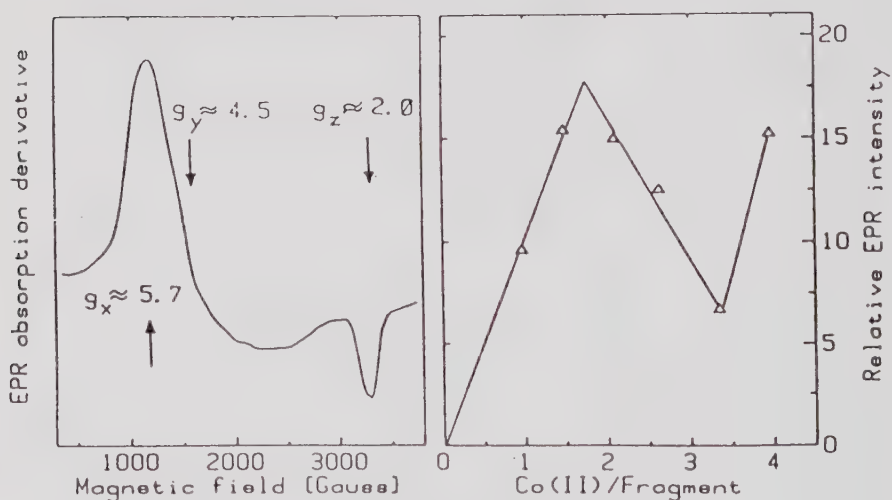


Figure 2-6: (Left) EPR spectrum of Co(II)-fragment containing 2.1 Co(II) per protein molecule. Conditions: 0.17 mM Co(II)-fragment in 160 mM Tris-HCl, pH 8.3; microwave power 0.8 mW; microwave frequency 9.236 GHz; 3.4 K. (Right) Dependence of the EPR signal intensity on the Co(II)-to-protein ratio. Signal amplitude was calculated employing the method of double integration.

CLUSTER FORMATION IN Cd(II)-FRAGMENT

To examine whether the unusual spectroscopic properties in the Co(II)-fragment is limited to this metal derivative similar metal titration studies were performed using Cd(II). Spectroscopic features of Cd(II)-MT are now well established (Vasak & Kägi, 1983, Gilg, 1985) and their interpretation is subject of another thesis (Willner, 1987). It is therefore not the purpose of this thesis to expand on the details of the spectral characteristics of this metal derivative. Only a brief description will be made of the titration of the α -fragment with Cd(II) using electronic absorption and CD techniques.

Electronic Absorption and CD Properties. Figure 2-7 shows the family of difference electronic absorption (top) and CD spectra (bottom) of the Cd(II)-fragment at different metal-to-protein ratios. Note that the spectrum of the apo-fragment at pH 8.3 is subtracted in the electronic absorption spectra. The first 2 Cd(II) equiv exhibit difference electronic absorption spectra with a maximum at 243 nm which undergoes a progressive red shift to 250 nm when the fully metal occupied Cd(II)₄-fragment is formed. This red shift was attributed to the participation of bridging thiolate sulfur ligands on binding in Cd(II)-MT and Cd(II)-substituted model peptides (Willner, 1987). The extinction coefficient in the Cd(II)₄-fragment at 250 nm was found to be $5200 \text{ M}^{-1} \text{ cm}^{-1}$ per sulfur atom, which is in close agreement with that measured in Cd(II)₇-MT.

While electronic absorption spectra of the Cd(II)-fragment at different metal-to-protein ratios can be compared with similar metal titration studies

performed earlier with Cd(II)-MT (Gilg, 1985; Willner, 1987) the development of the corresponding CD features shows some differences. Unlike in Cd(II)-MT, where a progressive intensity increase of the first low energy CD amplitude is discerned at all stages of filling, the Cd(II)-fragment undergoes a decrease in intensity at this CD band when the last Cd(II) equiv is getting bound (dashed line in Figure 2-7). CD is a highly sensitive spectral feature for differentiating between the clustered and nonclustered structure of Cd(II)-substituted MT. While isolated CdS_4^{2-} -sites show one CD band centered at (+) 245 nm the clustered structure (mixture of terminal and bridging thiolate ligands) elicits two oppositely signed CD bands. This band splitting is probably due to excitonic coupling of the bridging thiolate ligands and thus indicative of the clustered structure (Willner, 1987). The occurrence of the clustered structure in Cd(II)-MT during the course of metal titration is pH dependent. At neutral pH the clustered structure is formed preferentially whereas at $\text{pH} > 8$ isolated CdS_4^{2-} -complexes are more stable and, hence, are formed in the initial step of titration (Willner, 1987). Since in our case the titration of the fragment with Cd(II) was performed at pH 8.3 a nonclustered complex is formed first. However, formation of the clustered structure sets in before the second Cd(II) equiv is added (Figure 2-7, bottom). Thus, the cluster formation in Cd(II)-fragment resembles closely that observed in Co(II)-fragment (see above). To what degree the coordination geometry of the last metal binding site in the Cd(II)-fragment is also different still remains to be established, however. One method to learn more about the properties of Cd(II)-substituted α -fragment would be ^{113}Cd -NMR spectroscopy.

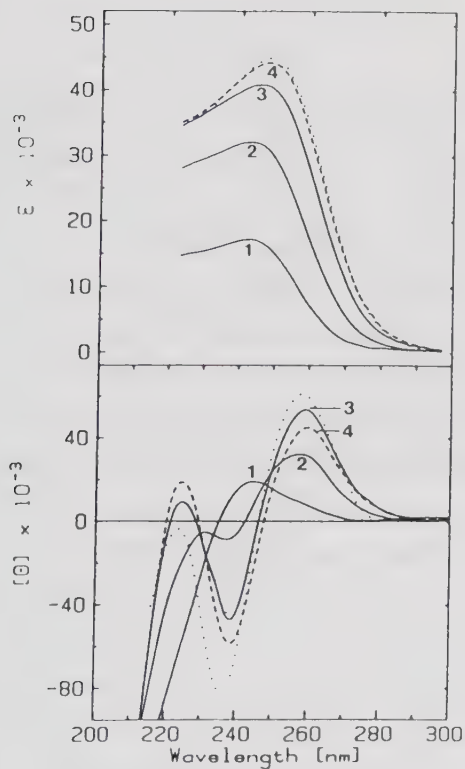
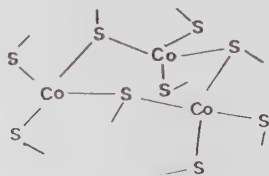


Figure 2-7: Electronic absorption (top) and CD (bottom) spectra of Cd(II)-fragment as a function of Cd(II)-to-protein ratio. The numbers indicate the moles of Cd(II) per mole of apofragment. The dashed lines represent the fully occupied Cd(II)₄-fragment. The dotted lines represent the spectrum obtained in the presence of an excess of Cd(II), i.e. 5 equiv of Cd(II) per fragment. Conditions: 19 μ M Cd(II)-fragment (protein concentration) in 10 mM Tris-Cl, pH 8.3. ϵ and $[\theta]$ are based on the protein concentration.

2-3 Conclusion

The spectroscopic studies presented here on the Co(II)-substituted α -fragment from rabbit liver MT-1 extend our knowledge of the structure of the metal binding sites in this protein. The stepwise incorporation of the first 3 Co(II) equiv into the apofragment results in spectral features which confirm the prevalence of distorted tetrahedral tetrathiolate Co(II) complexes. The essential preservation of the $\nu_3[{}^4A_2 \rightarrow {}^4T_1(P)]$ and $\nu_2[{}^4A_2 \rightarrow {}^4T_1(F)]$ transition in the course of the filling-up process documents that this geometry applies to the first three metal sites to be occupied. In contrast, the lesser changes in the ν_3 -transition of the electronic absorption and in the MCD spectrum upon binding of the fourth Co(II) ion and the emergence of a new low-intensity difference band in the near infrared region implies a different coordination geometry for this last metal site.

The electronic absorption, MCD and EPR spectra at various stages of filling suggest that the first two Co(II) equiv occupy non-interacting sites, each made up of four terminal thiolate ligands. As evidenced by the signal loss in the EPR spectrum and the pronounced red shift in the charge-transfer region of the electronic absorption spectrum, binding of the third Co(II) equiv brings about cross-linking among the metal binding sites via bridging thiolate ligands. A model compatible with all spectroscopic features of such a structure is a cyclic trinuclear metal thiolate cluster with a ratio of three Co(II) to nine thiolate sulfurs.



This structure resembles the NH_2 -terminal three-metal thiolate cluster (Cluster B) of mammalian MT's proposed by Otvos & Armitage (1980). The same model could allow the fourth metal to dock on the trinuclear metal thiolate cluster thereby forming an additional binding site of different coordination geometry.

Analogous differences between the α -fragment and the uncleaved protein are also seen in the Cd(II)-derivatives. Titration of the apofragment with Cd(II) ions revealed CD features which are not observed in metal titration of apoMT. Thus, the differences between the spectroscopic behaviour of the α -fragment and that of MT offer proof that the N-terminal segment (β -fragment) of MT is required to condition the formation of the C-terminal four-metal cluster in MT.

CHAPTER 3

IRON(II)-SUBSTITUTED METALLOTHIONEIN: EVIDENCE FOR THE EXISTENCE OF IRON-THIOLATE CLUSTERS

3-1 Introduction

Iron, one of the most abundant essential trace elements in biological systems, plays a fundamental role in many enzymes and other proteins. At the present time very little is known about intracellular iron metabolism (for a review see Hughes, 1978; Crichton, 1985). Although the major iron binding protein in cells is ferritin other high and low molecular iron binding species were identified (May et al., 1978). It is noteworthy that administration of iron induces not only the biosynthesis of apoferritin (Zähringer et al., 1975) but also of hepatic Zn(II)-MT (McCormick, 1984). That MT may serve a role in intracellular iron metabolism has been considered ever since it was first identified as a minor constituent in equine renal MT (Kägi & Vallee, 1961). However, because of the failure to observe in vitro binding of Fe(II) to apoMT this hypothesis has been questioned (Kojima et al., 1982). In this Chapter spectroscopic evidence is presented showing that under appropriate conditions Fe(II) forms equally well defined complexes with apoMT as do Zn(II), Cd(II) or Co(II). This finding leads to new perspectives for a possible role of MT in iron metabolism. My investigations focus on the optical properties of Fe(II)-MT and demonstrate the potential use of Fe(II) as a spectroscopic probe in this system.

3-2 Results and Discussion

(A) **Electronic Absorption Properties.** To determine the binding capacity of this protein towards Fe(II) the stepwise incorporation of Fe(II) into apoMT was investigated. Figure 3-1 shows the family of electronic absorption spectra of Fe(II)-MT of increasing intensity formed at various Fe(II)-to-protein molar ratios. At exactly 7 Fe(II) equiv bound the increase in absorbance in both near-UV and near-infrared regions levels off indicating formation of fully saturated Fe(II)₇-MT (Figure 3-2, top). Similar conclusions can be drawn also from the corresponding MCD studies (see below). At all stages of filling the coordination of the protein-bound Fe(II) remains tetrahedral as found for Co(II)-reconstituted MT (see Chapter 1). This is documented in particular by the occurrence of the characteristic ligand field absorption in the near-infrared region (Figure 3-1, right). Although the Fe(II)-dependent electronic absorption profile is obscured in part by vibrational transitions of the solvent, its center can be located at about 1850 nm with a molar extinction coefficient of $100 \text{ M}^{-1} \text{ cm}^{-1}$ per Fe(II). Similar absorption profiles in the near-infrared region have been reported for a number of inorganic mononuclear and polynuclear tetrahedral tetrathiolate Fe(II) complexes (Holah & Coucouvanis, 1975; Lane et al., 1977; Coucouvanis et al., 1981; Hagen et al., 1981) as well as for Fe(II)-complexes with peptide models (Anglin & Davison, 1975; Ueyama et al., 1985). In accordance with these studies, the ligand field absorption in Fe(II)-MT is attributable to the $^5\text{E} \rightarrow ^5\text{T}_2$ transition in the tetrahedral field which corresponds to the electronic promotion $e_{t_2}^{3,3} \rightarrow e_{t_2}^{2,4}$. The appearance of a rather strong absorption in the D₂O window between 2200 and 2400 nm

when compared with that at 1850 nm suggests, in agreement with the corresponding Fe(II) model compounds (Lane et al., 1977), the presence of an additional low energy electronic absorption band arising from the Fe(II)-thiolate complex. The relatively sharp absorption band at 2380 nm superimposed on the broad electronic band can be attributed to vibrational overtone transitions either from the protein or from residual HOD. The existence of more than one ligand field band in Fe(II)-MT suggests a departure from purely tetrahedral symmetry in agreement with a splitting of the t_2 orbitals. Independent support for this conclusion can be drawn from the ligand field spectrum of Co(II)-MT where the occurrence of a large energy separation within the characteristic triplet of the ν_3 -transition indicates distorted tetrahedral Co(II) coordination (see Chapter 1). On the basis of these data it is not possible to determine the ligand field splitting parameter experimentally as discussed in the following section.

A tetrahedral ligand field splits the 3 d orbitals into a lower degenerate pair e (d_{z^2} , $d_{x^2-y^2}$) and a upper triply degenerate set t_2 (d_{xy} , d_{xz} , d_{yz}) (for ligand field theory see Schläfer & Gliemann, 1980). The energy separation between the e and t_2 orbital levels corresponds to the ligand field splitting parameter Δ_t . This energy splitting for tetrahedrally coordinated first row transition metal ions is not large enough to cause spin pairing, an effect which is independent of the nature of the ligands. Consequently, five out of six 3d electrons of Fe(II) are spherically distributed among the five 3d orbitals and the sixth is equally distributed between the lower degenerate e (d_{z^2} , $d_{x^2-y^2}$) orbitals. Therefore, the electronic configuration of Fe(II)-MT in the ground state is $e^3 t_2^3$, which corresponds to the 5D state. Bair and Goddard (1978) have calculated the

energy levels of all five possible quintet states in the $\text{Fe}(\text{SH})_4^{2-}$ model compound. They found that all five states are nondegenerate, with d_z^2 as the lowest energy level and, therefore, orbitally doubly occupied. Taking the average of the two e and three t_2 components yielded a ligand field splitting, Δ_t , of 3500 cm^{-1} (Bair & Goddard, 1978). These authors also pointed out that the transitions from d_z^2 to the three t_2 levels should have almost equal intensities. As documented in Chapter 1 the ligand field splitting parameter of Co(II)-MT was estimated to be 4700 cm^{-1} (i.e. a transition should be expected at 2130 nm). In general Co(II)-complexes show slightly larger values than Fe(II)-complexes . Consequently, the spectral parameter Δ_t of Fe(II)-MT cannot be observed as the third d-d transition is expected to appear above 2500 nm.

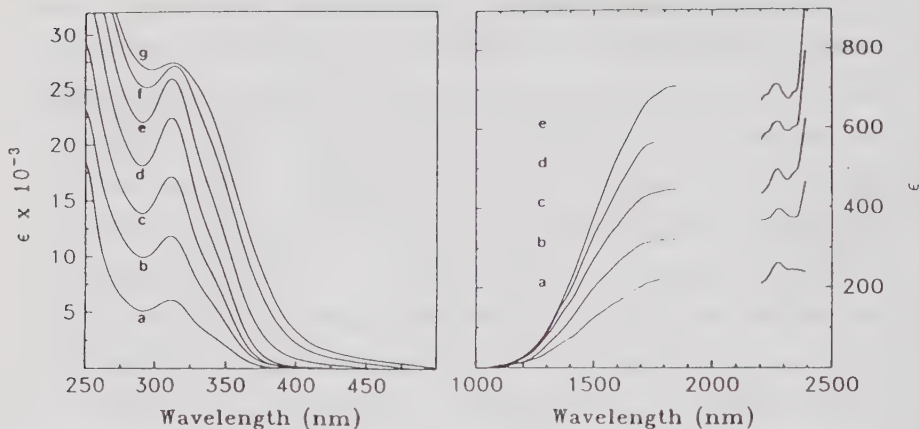


Figure 3-1: (Left) Absorption spectra in the charge-transfer region of Fe(II)-MT as a function of metal-to-protein ratio. The moles of Fe(II) per mole of apoMT are: a, 1; b, 2; c, 3; d, 4; e, 5; f, 6; g, 7. Conditions: 0.17 mM Fe(II)-MT in 160 mM Tris-DCl, pH 8.5, at room temperature; path length 2 mm. (Inset, left) Dependency of the absorbance ratio at 340 and 370 nm on the Fe(II)-to-protein ratio. (Right) Absorption spectra in the near-infrared region of the Fe(II)-MT as a function of metal-to-protein ratio. The moles of Fe(II) per moles of apoMT is: a, 2.3; b, 3.4; c, 4.6; d, 5.7; e, 6.9. Conditions: 0.68 mM Fe(II)-MT in 180 mM Tris-DCl pH 8.4, at room temperature; path length 10 mm. ϵ is based on the protein concentration.

From the 7-to-20 ratio of Fe(II)-to-cysteine residues a metal-thiolate cluster structure is inferred. More direct support for such aggregates is afforded by the spectroscopic changes in the UV region attending the metal incorporation (Figure 3-1 left). Since aromatic amino acids are lacking in MT (Vasak & Kägi, 1983) the electronic absorption profile in the near-UV region arises exclusively from metal binding. This is clearly seen from the rise in the absorption envelopes upon incremental addition of Fe(II). These spectral features which are characterized at low metal occupancy by an absorption maximum at 311 nm and a shoulder at 333 nm (Figure 3-1, left) match those found for reduced rubredoxin from *Clostridium pasteurianum*, a protein for which tetrahedral Fe(II) coordination by four cysteine thiolates is well documented (Eaton & Lovenberg, 1973; Watenpaugh et al., 1979). Bands and/or shoulders at similar positions have been observed also for a number of model complexes possessing overall tetrahedral tetrathiolate Fe(II) coordination (Anglin & Davison, 1975; Lane et al., 1977; Ueyama et al., 1985). According to Bair & Goddard (1978) these transitions were attributed to metal ligand charge-transfer transitions. Above about 4 Fe(II) equiv the absorption increments accompanying Fe(II) binding to apoMT become progressively smaller and a red shift of the charge-transfer envelope sets in leading to a loss of resolution. An illustration of this effect is given by the plot of the absorbance at 360 nm as a function of Fe(II)-to-apoMT ratio which is characterized by the change of the slope at about 4 Fe(II) equiv (Figure 3-2, top). As documented by the electronic absorption spectra of well-defined mononuclear and oligonuclear inorganic tetrahedral tetrathiolate Fe(II) model complexes, this red shift reflects most likely the formation of bridging thiolate ligands. Thus, both the binuclear $[\text{Fe}_2(\text{S-o-yl})_3]^{2-}$ and

the tetranuclear adamantane-type $[(\text{FeSPh})_4(\mu_2\text{-SPh})_6]^{2-}$ complexes display in the charge-transfer region a characteristic red shift with respect to the location of the corresponding mononuclear complexes (Lane et al., 1977; Coucouvanis et al., 1981; Hagen et al., 1981). The occurrence of the red shift in the course of the filling-up process of the metal binding sites of apoMT with Fe(II) would then indicate the progressive transition of some nonbridging thiolate ligands into bridging ones resulting in the formation of a clustered structure.

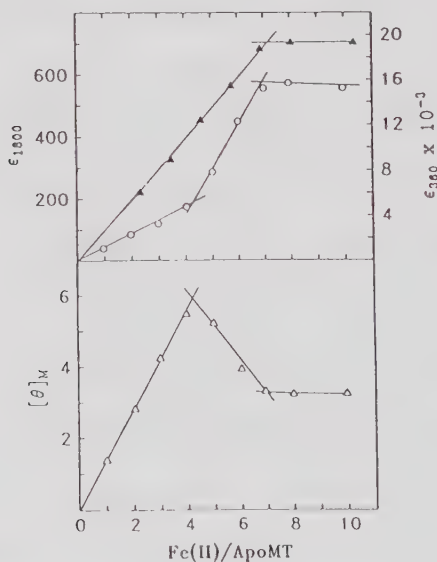


Figure 3-2: Dependency of the absorbance at 360 nm (o) and 1800 nm (▲) (top) and of the magnitude of the first low energy MCD maximum (Δ) (bottom) on the Fe(II)-to-apoMT ratio. Conditions as in Figure 3-1.

(B) MCD Properties. Since MCD was very informative in the studies of iron-sulfur proteins (Johnson et al., 1982) this technique was used to monitor the spectral changes attending the transition from the nonclustered to the clustered structure in Fe(II)-MT. At low metal occupancy the MCD profiles display a maximum at 332 nm and a trough and shoulder at 299 and 288 nm, respectively (Figure 3-3). The observed MCD profiles resemble those found in the mononuclear tetrahedral tetrathiolate Fe(II)-complexes of the reduced rubredoxins from *C. pasteurianum* and *D. gigas* as well as that of desulforedoxin of the latter species (Eaton & Lovenberg, 1973; Johnson et al., 1982). The MCD features in the former case have been suggested to mainly arise from B- or C-terms although a contribution of A-terms to the high energy MCD band has not been ruled out (Eaton & Lovenberg, 1973). The presence of C-terms has been confirmed by Johnson et al. (1982) who recently measured the MCD spectra of reduced rubredoxin and desulforedoxin, both from *D. gigas*, as a function of temperature down to 17.5 K. In correspondence to the electronic absorption behaviour of Fe(II)-MT (see above) substantial spectral changes above 4 Fe(II) equiv bound are also seen in the MCD spectra. They are characterized both by a similar red shift, mainly of the low-energy MCD band, and by a substantial amplitude decrease. An illustration of the latter effect is given by the plot of the molar magnetic ellipticity of the first low-energy MCD band as a function of Fe(II)-to-apoMT ratio (Figure 3-2, bottom). This effect is thought to reflect changes in electronic states upon cluster formation that are brought about by exchange coupling of vicinal Fe(II) via bridging thiolate ligands. Attempts to document such exchange coupling by EPR measurements in Fe(II)-MT at 4 K remained unsuccessful. This may be

attributed to the persistence of the large zero-field splitting in both oligonuclear and mononuclear complexes of Fe(II). Since the MCD signal of paramagnetic centers are temperature dependent (C-term) whereas those of diamagnetics are temperature independent (Johnson et al., 1982) the decrease of the MCD signal can be attributed to a partial loss of the C-term when the Fe(II) ions interact antiferromagnetically upon cluster formation. To gain more insight into this phenomenon a mixed metal Fe(II)₁Zn(II)₆-MT was prepared. If the intensity drop of the MCD signal is mainly due to exchange coupling one would expect a similar MCD amplitude in both Fe(II)₁-MT and Fe(II)₁Zn(II)₆-MT, respectively. Indeed, the larger amplitudes of the MCD signals in Fe(II)₁Zn(II)₆-MT compared to Fe(II)₇-MT supports partially this hypothesis (Figure 3-5, bottom). In the former species the first low energy band is approximately 60% larger than that of the latter one (Table V). However, the magnetic ellipticity at 332 nm of Fe(II)₁-MT is still about 40% more intense than that of Fe(II)₁Zn(II)₆-MT. A possible explanation for this discrepancy is the difference in the nature of the ligands. The presence of vicinal Fe(II) ions in Fe(II)₁Zn(II)₆-MT is unlikely, in agreement with similar studies of Co(II)₁Zn(II)₆-MT (see Chapter 1).

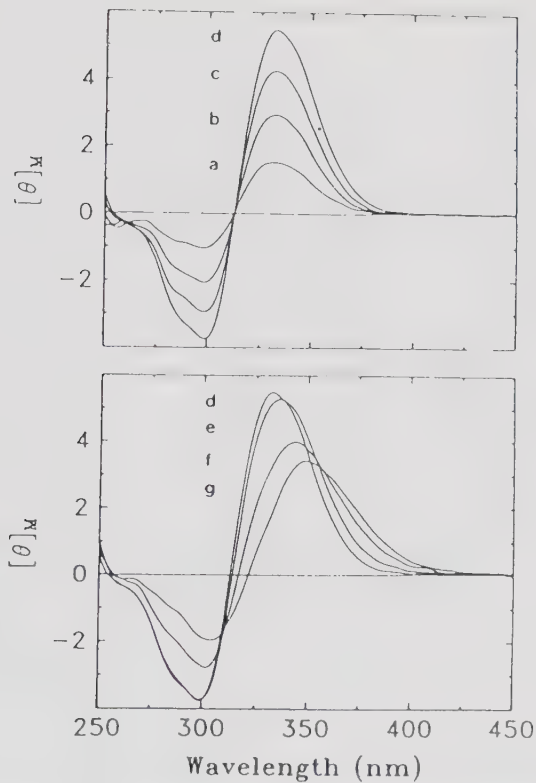


Figure 3-3: MCD spectra of Fe(II)-MT as a function of metal-to-protein ratio. The moles of Fe(II) per mole of apoMT are: a, 1; b, 2; c, 3; d, 4; e, 5; f, 6; g, 7. Conditions as in Figure 3-1, left. $[\theta]_M$ is based on the protein concentration.

Table V Spectral Data of tetrahedral tetrathiolate Fe(II)-Complexes in MT

Complex	$^5E \rightarrow ^5T_2$ a	$S \rightarrow Fe(II)$ charge transfer ^b
Fe ₁ -MT	abs ^c 1850 (0.1)	311 (1.47) 333,sh (0.88)
	CD ^d n.d.	260 (3.26) 280,sh (1.88) 314 (-1.84) 346 (1.36)
	MCD ^e n.d.	285,sh (-0.21) 298 (-0.26) 332 (0.38)
Fe ₇ -MT	abs 1850 (0.1)	314,sh (1.35)
	CD n.d.	274,sh (0.47) 300 (2.00) 329 (0.82) 351 (-0.45) 404 (0.10)
	MCD n.d.	285,sh (-0.05) 304 (-0.10) 349 (0.17)
Fe ₁ Zn ₆ -MT	abs n.d.	314,sh (1.26)
	CD n.d.	287 (-1.36) 306 (4.39) 320,sh (2.50) 340 (-4.46) 376 (-0.50)
	MCD n.d.	278,sh (-0.04) 308 (-0.13) 343 (0.27) 370,sh (0.13)

a) Values are calculated per iron atom

b) Values are calculated per sulfur atom

c) In nm ($\epsilon \times 10^{-3}$, M⁻¹ cm⁻¹)

d) In nm ($\theta \times 10^{-3}$, deg cm² dmol⁻¹)

e) In nm (M , deg cm² dmol⁻¹ Gauss⁻¹)

n.d. not determined

(C) CD Properties. Differences between the nonclustered and clustered structure of Fe(II)-MT manifest themselves also in the corresponding CD spectra (Figure 3-4). Up to binding of the first 4 equiv of Fe(II) the CD profile increases in intensity without changing shape. The band extrema at (+) 346 and (-) 314 nm which were also reported for reduced rubredoxin (Eaton & Lovenberg, 1973) and Fe(II) peptide model complexes coordinated solely by cysteine sulfur (Ueyama et al., 1985) are typical of isolated FeS_4^{2-} -sites in Fe(II)-MT. Another extremum at 260 nm and a shoulder near 280 nm can be attributed to charge-transfer excitations. Subsequent addition of Fe(II) up to fully metal occupied $\text{Fe(II)}_7\text{-MT}$ alters the CD features markedly. The extrema at (-) 351 and (+) 349 nm changes their sign and a new low intensity CD band emerges at (+) 404 nm. Based on molecular orbital calculations of Fe(SH)_4^{2-} (Bair & Goddard, 1978) this weak band may be assigned to the spin-forbidden quintet $\rightarrow$ triplet d-d transition. Further addition of Fe(II) to the fully occupied $\text{Fe(II)}_7\text{-MT}$ has no effect on the CD spectrum implying that additional Fe(II) does not bind to $\text{Fe(II)}_7\text{-MT}$. Interestingly, the CD bands of $\text{Fe(II)}_1\text{Zn(II)}_6\text{-MT}$ display the same inversion in CD sign as do those of $\text{Fe(II)}_7\text{-MT}$ when compared to the nonclustered structure (Figure 3-5).

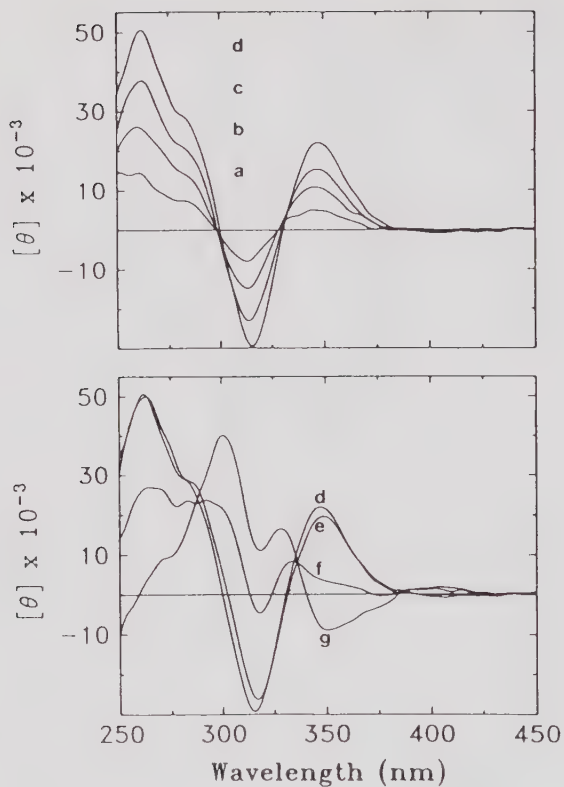


Figure 3-4: CD spectra of Fe(II)-MT as a function of metal-to-protein ratio. The moles of Fe(II) per mole of apoMT are: a, 1; b, 2; c, 3; d, 4; e, 5; f, 6; g, 7. Conditions as in Figure 3-1, left. $[\theta]$ is based on the protein concentration.

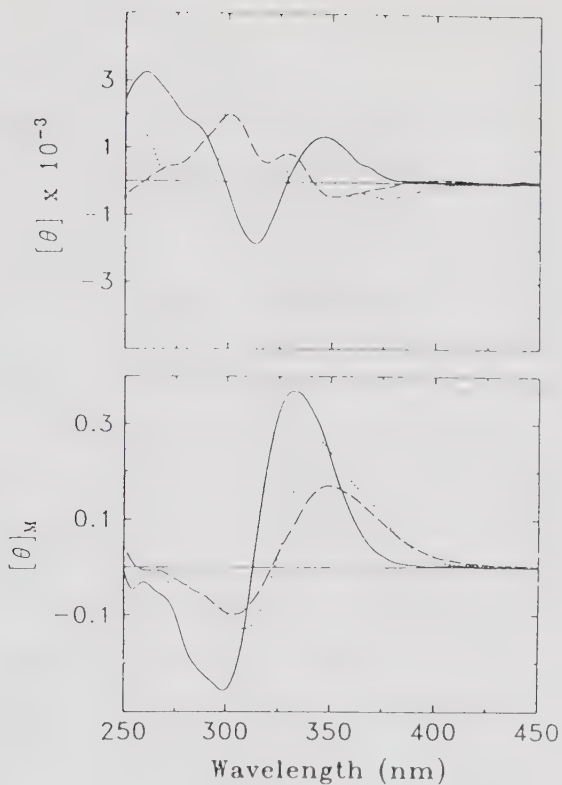


Figure 3-5: CD (top), and MCD (bottom) spectra of $\text{Fe(II)}_1\text{-MT}$ (—), $\text{Fe(II)}_7\text{-MT}$ (---), and $\text{Fe(II)}_1\text{Zn(II)}_6\text{-MT}$ (·····). $[\theta]$ and $[\theta]_M$ are calculated per sulfur atom. Conditions: 160 mM Tris-Cl, pH 8.3 at room temperature.

3-3 Conclusion

The spectroscopic data presented document the chemical existence of $\text{Fe(II)}_7\text{-MT}$ containing Fe(II) -thiolate clusters similar to those formed by Zn(II) and Cd(II) in the native protein. The existence of this species in vivo seems rather unlikely, however, because of its extreme air sensitivity and the instability of the resulting oxidation product in aqueous solution. All iron-sulfur cluster proteins found thusfar in biological systems contain besides cysteine also inorganic sulfide as ligands. It is conceivable that this extrinsic ligand is a stabilizing factor in iron-sulfur clusters.

These data illustrate also the potential use of Fe(II) as a spectroscopic probe for metal binding sites in metalloproteins having cysteine as ligand. While the electronic absorption features in the near-infrared region provide primarily information concerning the coordination geometry of the metal binding site, those in the charge-transfer region are more indicative of the chemical nature of these ligands. CD and MCD data of this region are sensitive indicators for distinguishing between terminal and bridging thiolate ligands. Inversion of the sign of the CD bands and reduction of the MCD signals are characteristic features of the transformation of Fe(II) -complexes from the nonclustered to the clustered forms. That the magnitude of both low energy MCD bands are also influenced by magnetic exchange interaction between Fe(II) ions is demonstrated by the fact that MCD signals in $\text{Fe(II)}_7\text{-MT}$ are much smaller than in $\text{Fe(II)}_1\text{Zn(II)}_6\text{-MT}$. Stepwise incorporation of Fe(II) into apoMT shows also that the first 4 equiv of Fe(II) enter separate metal binding sites each composed of four tetrahedrally arranged terminal thiolate ligands and that the clusters are

formed only when more Fe(II) is added. The same mode of metal incorporation was previously shown for Co(II)-MT. Since ^{57}Fe is a suitable isotope for Mössbauer spectroscopy Fe(II)-MT might provide additional information about this metal derivative.

EXPERIMENTAL PROCEDURES

Chemicals, Instruments and Physical Measurements. Rabbit liver MT was isolated from rabbits injected with CdCl_2 by 20 subcutaneous injections of 1 mg of cadmium sulfate per kg body weight at intervals of 2-3 days. MT was purified by a combination of the procedures of Kimura et al. (1979) and Kägi et al. (1974). The purity of each preparation was examined by amino acids analysis (Durrum D-500) and by metal analysis using atomic absorption spectrometry (Instrumentation Laboratory IL 157). All studies reported in this work were performed on the isoform MT-1. All inorganic and organic chemicals were of reagent grade or better and prepared solutions were stored in polyethylene bottles. Subtilisin Carlsberg from *Bacillus subtilis* was from Fluka. Absorption spectra were recorded on a Perkin-Elmer model 340 spectrophotometer using 1.0, 0.2 and 0.1 cm quartz cells. The spectra in the near infrared region were obtained in solutions of D_2O (99.8%). Molar absorptivities ϵ are given in units of $\text{M}^{-1} \text{cm}^{-1}$. EPR measurements were performed at a temperature of 3.4 K using a Varian E 112 spectrometer operating in the X-band mode. EPR spectra were quantitated by the method of double integration (Palmer, 1967). A Jasco spectropolarimeter (model J-500) connected on line with an Epson-QX personal computer was used for MCD- and CD-measurements employing a constant magnetic field of 15 kG at room temperature. For each measurement the field was set alternately parallel and antiparallel in the direction of the light beam, a procedure which improves the signal to noise ratio by a factor $2^{1/2}$. MCD and CD curves were calculated algebraically from the

measured differences in absorbance, $\Delta E(+)$ and $\Delta E(-)$, of left and right circularly polarized light for the parallel (+) and antiparallel (-) direction of the magnetic field H, respectively. For the two directions the signals are given by the expressions:

$$\Delta E(+) = \Delta E(\text{CD}) + [H] \Delta E(\text{MCD})$$

and

$$\Delta E(-) = \Delta E(\text{CD}) - [H] \Delta E(\text{MCD})$$

Taking the difference of the two observed signals cancels the natural ellipticity $\Delta E(\text{CD})$ and leaves the magnetic ellipticity $\Delta E(\text{MCD})$ at twice the intensity of a single measurement. Addition of the two observed signals yields twice the natural ellipticity. Molar ellipticities $[\Theta]_M$ and $[\Theta]$ are given in units of $\text{deg cm}^2 \text{ dmol}^{-1} \text{ G}^{-1}$ and $\text{deg cm}^2 \text{ dmol}^{-1}$, respectively. $[\Theta]$ was calculated according to the equation $[\Theta] = (\Theta/10\text{cd})$. Θ is the ellipticity measured directly in millidegrees, $(\Theta = 3300(A_L - A_R))$, c is the molar concentration and d is the pathlength in cm. $[\Theta]_M$ was normalized to 1 G.

Preparation of α -Fragment. The procedure of Winge and Miklossy (1982) was used with minor modifications. Prior to subtilisin cleavage 2 ml of 1.25 mM apoMT (15 mg) in 0.01 N HCl was used for partial reconstitution with 4 equiv of CdCl_2 . Subsequently, this solution was diluted with 3 ml 10 mM

potassium phosphate buffer (pH 7.6) and the pH adjusted to 7.6 with 1 N KOH. 100 μ l of a freshly prepared solution of subtilisin (2 mg/ml) in the same buffer was then added and the sample incubated at 4° C for about 15 h. In order to minimize oxidation of cysteine sulfur all solutions were saturated prior to use with nitrogen and all preparative steps were carefully performed in a N₂-purged glove-box. Subsequently the sample was concentrated to about 1.5 ml by ultrafiltration (Amicon apparatus, YM2 membrane) and separated over a Sephadex G-75 superfine column (150 x 1.5 cm) equilibrated with 10 mM phosphate buffer pH 7.6, at 4° C. The elution profile consisted of two well separated peaks with an elution coefficient K_D of 0.51 and 0.68, respectively. On the basis of the amino acid composition and N-terminal amino acid determination (De Jong et al., 1982) the peak with $K_D=0.68$ contained the C-terminal fragment of rabbit-liver MT-1 (residues 31-61) starting with Lys-31. The preparative yield in nine independent preparations ranged between 35-40%. The metal-free peptide (apofragment) was prepared similarly to apoMT (see below). Prior to metal reconstitution all eleven cysteine residues were determined using Ellman's reagent (DTNB) in 10 mM potassium phosphate buffer pH 7.5 ($\epsilon_{412} = 13'600 \text{ M}^{-1} \text{ cm}^{-1}$) (McGilvray & Morris, 1971). On the basis of quantitative amino acid analysis the molar extinction coefficient of the α -fragment at 220 nm in 0,01 N HCl was found to be $32'400 \text{ M}^{-1} \text{ cm}^{-1}$.

Preparation of ApoMT. The metal-free protein, apoMT, was prepared by gel filtration of the native form at low pH. A neutral solution of (Zn,Cd)-MT was adjusted to pH 1 and subsequently passed over a Sephadex G-25

column, equilibrated with 0.01 N HCl. The pooled fractions were diluted (1:1) with bidistilled water, lyophilized and stored at -20° C. For experiments in the near-infrared region ($\lambda > 1000$ nm) the lyophilized protein was dissolved in 0.01 N DCl for about 15 minutes, diluted with D_2O (1:1) and lyophilized again. This procedure was repeated twice in order to minimize residual HOD in the protein. Protein concentration was determined spectrophotometrically by measuring the absorbance of apoMT in 0.01 N HCl at 220 nm using an extinction coefficient of $48'200 \text{ M}^{-1} \text{ cm}^{-1}$ (Bühler & Kägi, 1979) or by quantitative amino acid analysis. Prior to metal reconstitution all cysteine residues were determined using Ellmann's reagent (DTNB).

Preparation of Co(II)-MT and Co(II)-Fragment. All solutions used in the preparation of Co(II)-MT and the Co(II)-fragment were degassed on a vacuum line prior to use and the procedures were performed in an argon-purged glove-box. A freshly prepared solution of 0.2 mM apoMT or apofragment in 0.1 N HCl was mixed with 50 mM $CoSO_4$ in bidistilled water yielding the desired metal-to-protein ratio. The solution mixture was then adjusted to pH 8.3 by the addition of 2 equiv of 1 M Tris-base (Trizma from Sigma). The metal-to-protein ratios were determined independently using a small aliquot of the sample. The green coloured Co(II)-complex is highly oxygen sensitive and yields a light brown product upon exposure to air. For measurements in the near infrared region D_2O was used as solvent.

$Co(II)_1Zn(II)_6$ -MT and $Co(II)_1Cd(II)_6$ -MT were prepared in two ways. First, by the addition of 1 equiv of Co(II) and 6 equiv of Zn(II) or Cd(II) to a 0.4 mM solution of apoMT in 0.1 N HCl, respectively. This solution

mixture was then adjusted to pH 8.3 by the addition of 2 equiv of 1 M Tris-base. All samples prepared in that way were kinetically stable, i.e., no changes in electronic absorption could be monitored during several hours. Second, by the addition of 1 equiv of Co(II) to a neutralized solution of $\text{Cd(II)}_6\text{-MT}$ or $\text{Zn(II)}_6\text{-MT}$ at pH 8.3. These Co(II)-substituted species exhibited similar electronic absorption profiles but were kinetically unstable. $\text{Co(II)}_1\text{Cd(II)}_6\text{-MT}$ changed its electronic absorption properties more rapidly compared to $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$. Therefore, all samples of $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ and $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ reported in Chapter 1 were prepared according to the first procedure.

Preparation of Cd(II)-Substituted α -Fragment. 100 μl of a solution of 0.5 mM apofragment in 0.01 N HCl was added to a buffer-solution of 2.5 ml 10 mM Tris-HCl, pH 8.3. For each titration step (1 equiv of Cd(II)) 10 μl of 5 mM CdCl_2 was added, yielding the desired stoichiometric amount. To avoid oxidation of cysteine sulfur all solutions were degassed on a vacuum line prior to use and the procedures were performed in a nitrogen purged glove-box.

Preparation of Fe(II)-MT. Fe(II)-MT was prepared similar to Co(II)-MT (see above) using $(\text{NH}_4)_2\text{Fe(SO}_4)_2 \cdot 6 \text{H}_2\text{O}$ salt. At neutral pH $\text{Fe(II)}_7\text{-MT}$ has a yellow color and is extremely air sensitive. On exposure of the aqueous solution to air, the color changes immediately to wine-red and a slow precipitation of protein sets in.

$\text{Fe(II)}_1\text{Zn(II)}_6\text{-MT}$ was obtained by the procedures used for the preparation of $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$ (see above). However, in this case the electronic absorption spectrum of $\text{Fe(II)}_1\text{Zn(II)}_6\text{-MT}$ remained unchanged within the time period studied (1/2 h) when 1 Fe(II) equiv was added to a solution of $\text{Zn(II)}_6\text{-MT}$ at pH 8.3. Preparation by the first procedure, i.e. addition of 1 Fe(II) equiv plus 6 equiv of Zn(II) to a solution of 0.2 mM apoMT in 0.1 N HCl and adjustment to pH 8.3 resulted in an $\text{Fe(II)}_1\text{Zn(II)}_6\text{-MT}$ species whose electronic absorption spectrum underwent a slight change with time. The preparation of $\text{Fe(II)}_1\text{Zn(II)}_6\text{-MT}$ was less quantitative than that of $\text{Co(II)}_1\text{Zn(II)}_6\text{-MT}$. At least 3 Fe(II) equiv had to be added to $\text{Zn(II)}_6\text{-MT}$ in order to reach a plateau in the absorption profile. Nonspecific binding of Fe(II) ions to the protein molecule was excluded on account of the absence of any manifestation in the electronic absorption spectra when an excess of Fe(II) was added to $\text{Zn(II)}_7\text{-MT}$. All samples of $\text{Fe(II)}_1\text{Zn(II)}_6\text{-MT}$ reported in Chapter 3 were prepared by adding 3 equiv of Fe(II) to a solution of $\text{Zn(II)}_6\text{-MT}$ at pH 8.3.

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G L O S S A R Y O F S Y M B O L S
A N D A B B R E V I A T I O N S

Symbols

- $[\Theta]$, molar ellipticity [$\text{deg cm}^2 \text{ dmol}^{-1}$]
 $[\Theta]_M$, molar magnetic ellipticity [$\text{deg cm}^2 \text{ dmol}^{-1} \text{ G}^{-1}$]
 ϵ , extinction coefficient [$\text{M}^{-1} \text{ cm}^{-1}$]
 H , magnetic field [gauss]
 K_D , elution coefficient of gelfiltration
 T_d , tetrahedral
3D, 2D, three dimensional, two dimensional
 X_L , optical electronegativity of the ligand
 X_M , optical electronegativity of the metal ion
 Δ_t , ligand field strength
 B' , Racah parameter

Abbreviations

- ApoMT, apometallothionein
CD, circular dichroism
DTNB, 5,5'-dithiobis(2-nitrobenzoic acid)
EPR, electron paramagnetic resonance
MCD, magnetic circular dichroism
MT, metallothionein
NMR, nuclear magnetic resonance
Tris, tris(hydroxymethyl)-aminomethane

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